

*GRAPEVINE FANLEAF VIRUS: BIOLOGY, BIOTECHNOLOGY AND  
RESISTANCE*

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*GRAPEVINE FANLEAF VIRUS: BIOLOGY, BIOTECHNOLOGY AND  
RESISTANCE*

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*Grapevine fanleaf virus* (GFLV) causes fanleaf degeneration of grapevines. GFLV is present in most grape growing regions and has a bipartite RNA genome. The three goals of this research were to (1) advance our understanding of GFLV biology through studies on its satellite RNA, (2) engineer GFLV into a viral vector for grapevine functional genomics, and (3) discover a source of resistance to GFLV. This author addressed GFLV biology by studying the least understood aspect of GFLV: its satellite RNA. This author sequenced a new GFLV satellite RNA variant and compared it with other satellite RNA sequences. Forensic tracking of the satellite RNA revealed that it originated from an ancestral nepovirus and was likely introduced from Europe into North America. Greenhouse experiments showed that the GFLV satellite RNA has commensal relationship with its helper virus on a herbaceous host. This author engineered GFLV into a biotechnology tool by cloning infectious GFLV genomic cDNAs into binary vectors, with or without further modifications, and using *Agrobacterium tumefaciens* delivery to infect *Nicotiana benthamiana*. Tagging GFLV with fluorescent proteins allowed tracking of the virus within *N. benthamiana* and *Chenopodium quinoa* tissues, and imbuing GFLV with partial plant gene sequences proved the concept that endogenous plant genes can be knocked down. Infectivity of the viral vector depended on the identity of the GFLV strains or reassortants, on co-application of heterologous silencing suppressors and on lower ambient temperatures. No natural sources of resistance to GFLV exist within *Vitis* spp., but certain

herbaceous hosts such as *N. tabacum* (tobacco) are resistant. This author used tobacco, its wild relatives, and hybrids between tobacco and wild relatives to evaluate the genomic and physiological basis of resistance. Resistance to GFLV in tobacco is governed by systemic recovery from virus infection that is additively inherited and likely multi-allelic. This research has opened new avenues to understand virus and plant evolution, and furnishes geneticists with a new tool to functionally characterize host genes. This dissertation also includes a history of pathogen-derived resistance with specific reference to plant virus resistance.

## BIOGRAPHICAL SKETCH

John was raised in Lubbock, Texas and attended Texas Tech University as an undergraduate. At Texas Tech, he worked with Dr. Robert Wright on cotton breeding and biotechnology. John's research interests include transgenic technologies for plant stress resistance, breeding for durable plant resistance and plant functional genomics tools. John has been the first author/ co-author on seven publications and primary investigator or project director on two grants focused on plant biology and biotechnology. He has taken a variety of leadership roles in the Plant Pathology Graduate Student Association at Cornell including treasurer (2009-2011), the Student Association of the Geneva Experiment Station including garden coordinator (2010-2012), has led a collaborative Cornell colloquium (2012) and has coordinated invited lectures of multiple students and faculty from around the United States. John has served as *ad hoc* reviewer for *Crop Science* and *Transgenic Research*, assisted teaching the Cornell course Magical Mushrooms, Mischievous Molds, and has participated in extension work for grapevine virus control in the Finger Lakes. When John is not doing research, he enjoys pretending to fish with his friend Ben and dancing a very serious two-step with his beautiful wife Kelly.

Dedicated to Hussein Alzubi

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## PREFACE

*Grapevine fanleaf virus* (GFLV) is a small pathogen in size. It encodes only eight individual proteins and is encapsidated in 30nm particles, but its interactions with plant hosts are extraordinarily complex. This dissertation encompasses reviews and primary research of GFLV biology including its genetic diversity, host range, inoculation methods, evolutionary biology and uses in biotechnology. In Chapter 1, this author reviews GFLV biology and discuss its relationship with other viruses of the genus *Nepovirus*, family *Secoviridae*. In Chapter 2, this author discusses the natural history, evolutionary biology, and host and helper virus interactions of the nepovirus subgroup A satellite RNA. In Chapter 3, this author presents proofs-of-concept that GFLV is engineered into a vector for plant functional genomics and other uses. In Chapter 4, this author describes variables that are associated with reliable plant systemic infection when GFLV is inoculated through *Agrobacterium tumefaciens*. In Chapter 5, this author reviews the history of pathogen-derived resistance applied to viruses through 2009. In Chapter 6, this author presents an assessment of the GFLV host range within *Nicotiana* and a theory of how allopolyploids impact evolution of basal virus resistance. Finally, in Chapter 7, this author suggests research projects to better understand the GFLV satellite RNA, improve the GFLV vector, and an overview of how plant resistance to viruses can be improved. Readers of this dissertation will gain an appreciation of the complex yet elegant nature of GFLV biology and insights into broader issues in plant virology, viral vectors for plant functional genomics and plant resistance to viruses.



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## CHAPTER 1

### ***GRAPEVINE FANLEAF VIRUS AND FANLEAF DEGENERATION***

#### ***THE DISEASE***

*Grapevine fanleaf virus* (GFLV) is one of the most serious and widespread grapevine virus diseases. GFLV causes grapevines to produce lower fruit yield and reduced berry quality, misshapen leaves, shortened internodes, leaf yellowing, mottling and vein clearing (Andret-Link *et al.*, 2004). The economic impact on grapevine production systems is severe with \$1 billion annual losses to the French grape and wine industries (Andret-Link and Fuchs, 2005).

Based on the natural distribution of its highly specific ectoparasitic nematode vector, *Xiphinema index*, GFLV is thought to originate from the Caucasus region of East Asia (Raski *et al.*, 1983). *Vitis vinifera* (grapevine) was domesticated in its center of origin in Anatolia or the Caucasus by 4,000 B.C.E. and was carried to Mesopotamia and Egypt by 2,000 B.C.E., and France by 500 B.C.E. (Hancock, 2004). Although the ancient use of cuttings and layering probably hindered the dissemination of the nematode vector on a local level, long-distance grapevine transport would have required rooted plants and thus facilitated the worldwide dispersal of the nematode and associated virus. Today, GFLV is present in all major grape-growing regions except the Finger Lakes Region of New York State and other central and northeastern North American regions.

GFLV is one of several viruses that cause fanleaf degeneration. The other

viruses that are causal agents of fanleaf degeneration are related to GFLV and belong to the genus *Nepovirus* in the family *Secoviridae*. Based on genome homology and identity, nepoviruses are classified into subgroups A, B and C (Sanfaçon *et al.*, 2009). GFLV, *Arabidopsis mosaic virus* (ArMV), *Tobacco ringspot virus* (TRSV) and *Grapevine deformation virus* (GDefV) (Elbeaino *et al.*, 2012) are subgroup A nepoviruses that cause fanleaf degeneration. Subgroup B nepoviruses that cause fanleaf degeneration are *Tomato black ring virus* (TBRV) and *Grapevine chrome mosaic virus* (GCMV). A subgroup C nepovirus that causes fanleaf degeneration is *Tomato ringspot virus* (ToRSV) (Sanfaçon *et al.*, 2009).

The nepoviruses that cause fanleaf degeneration are systemically infective in grapevines. Asexual propagules (cuttings) taken from infected tissue contain the viruses, and thus the viral diseases will become established in vineyards where infected clones are planted or used for grafting. The disease may be present but not established in vineyards where infected clones were introduced in the absence of soil infested by the nematode vectors. This has been observed for GFLV (Gottula *et al.*, 2013) and ArMV (Celebi-Toprak *et al.*, 2013) in the U.S. Department of Agriculture Cold Hardy Grape Genetics Germplasm Repository in Geneva, NY. Conversely, aviruliferous *X. index* infesting vineyards will not spread fanleaf degeneration, and nematode parasitism can be effectively controlled using resistant rootstock material (Reisch *et al.*, 2011).

### **PATHOGEN BIOLOGY**

Nepoviruses share a common bipartite genome structure composed of single-

stranded positive-sense RNAs. The two nepovirus genomic RNAs are RNA1 and RNA2, each of which includes a virus-encoded protein covalently attached to its 5' end (VPg, viral protein, genome-linked) and a poly-A tail extending from its 3' end. Nepoviruses share common genome expression mechanisms with other members of *Picornavirales* including monocistronic translation and proteolytic processing of individual protein components (Sanfaçon *et al.*, 2009). Nepovirus subgroup A RNA1 contains coding regions for the 1A, 1B<sup>Hel</sup> (helicase), 1C<sup>VPg</sup>, 1D<sup>Pro</sup> (proteinase) and 1E<sup>RdRp</sup> (RNA-dependent RNA polymerase) proteins. The RNA2 of subgroup A nepovirus species contain coding regions for the 2A<sup>HP</sup> (homing protein), 2B<sup>MP</sup> (movement protein) and 2C<sup>CP</sup> (coat protein) proteins. Additionally, about one third of GFLV isolates from Europe and Asia contain a satellite RNA (Saldarelli *et al.*, 1993).

The function of GFLV protein 1A is poorly characterized, but is thought to form the structure of the nepovirus replication complex on ER membranes (Ritzenthaler *et al.*, 2002). The 1B<sup>Hel</sup> protein contains a nucleoside triphosphate-binding domain and is a putative helicase (Ritzenthaler *et al.*, 1991). Certain nepoviruses, including ArMV, show post-translational processing of the 1B<sup>Hel</sup> into a helicase and a hydrophobic protein (X2), which is a membrane anchor in the virus replication complex (Sanfaçon *et al.*, 2012). There is no evidence that GFLV produces multiple 1B<sup>Hel</sup> translation products (J. Gottula and C. Keichinger, unpublished data).

The identification of the GFLV silencing suppressor is pending (Vigne *et al.*, 2013), but may be the 1B<sup>Hel</sup>, which contains a GW (glycine-tryptophan) motif. GW motifs interact with Argonaute proteins to alter silencing suppression activity (Burgyan and Hazevelde, 2011; Garcia *et al.*, 2012) and can sometimes provide hints

as to the identity of viral suppressors of silencing, especially when multiple GW motifs are present and are present with WG motifs (El-Shami *et al.*, 2007). The GFLV GW motif within 1B<sup>Hel</sup> occurs without a WG motif and is fully conserved between GFLV and ArMV, but so are 71.7% of 1B<sup>Hel</sup> amino acids among the eight fully sequenced GFLV and ArMV isolates (J. Gottula, unpublished data). Most other sequenced nepoviruses contain one GW domain within 1B<sup>Hel</sup>, though the positions are not conserved among or within subgroups, and most 1B<sup>Hel</sup> proteins do not contain a WG motif (J. Gottula, unpublished data). The functional significance of the GW motif in GFLV 1B<sup>Hel</sup> is unknown.

Nepoviruses encode a VPg that is affixed to the 5' ends of (+) and (-) strand of GFLV RNAs including RNA1, RNA2 and the satellite RNA (Pinck *et al.*, 1988). VPg proteins interact with plant translation machinery to achieve either translation or replication (Jiang and Laliberté, 2011). Nepovirus VPgs are much smaller than those of other viral genera (Jiang and Laliberté, 2011). For ToRSV, the VPg exists in proteolytically unprocessed forms with the neighboring helicase, proteinase and the RdRp, and one of these unprocessed forms likely comprise the nepovirus primer for replication (Chisholm *et al.*, 2007). Like the VPg of potyviruses, the nepovirus VPg interacts with *eukaryotic Initiation factor 4E* (Léonard *et al.*, 2002), which could open up the interesting possibility of achieving nepovirus resistance through mutation of host *eIF4E* alleles (Charron *et al.*, 2008).

The proteinase of GFLV is a cysteine-like proteinase structurally related to chemotrypsin (Sanfaçon *et al.*, 2009). It recognizes the following residue combinations in GFLV: cysteine/arginine, arginine/glycine, glycine/serine and

glycine/glutamate (Ritzenthaler *et al.*, 1991; Margis *et al.*, 1994). Nine to 25 amino acids surrounding each of these residues are conserved in GFLV and ArMV and are probably necessary for proteinase recognition of these sites (J. Gottula, unpublished; Wetzel *et al.*, 2013). The proteinase functions on the RNA1 polyprotein *in cis* and RNA2 polyprotein *in trans* and may require the 1A as a cofactor (Ritzenthaler *et al.*, 1991).

The amino acid sequence of GFLV RdRp is highly conserved (Oliver *et al.*, 2010) and shows high identity to the ArMV RdRp (Gottula *et al.*, 2013). The RdRp shares four conserved amino acid motifs with other members of *Secoviridae* including a GDD (glycine-aspartate-aspartate) motif (Ritzenthaler *et al.*, 1991). The RdRp was recently found to be the GFLV symptom determinant in *N. benthamiana* and *N. clevelandii*, and the region of the RdRp that determines symptoms was mapped to the its 3'/C-terminal region upstream of the conserved GDD motif (Vigne *et al.*, 2013). Because this region is not post-translationally cleaved from the RdRp and does not appear to relate to higher GFLV accumulation (Vigne *et al.*, 2013), questions remain about whether symptoms relate to protein or RNA factors encoded by the symptom-producing GFLV strain GHu.

Little is known about the GFLV 2A<sup>HP</sup> protein other than it is necessary for RNA2 replication (Gaire *et al.*, 1999). The 2A<sup>HP</sup> protein shows relatively high amino acid diversity among GFLV isolates (Oliver *et al.*, 2010), though less interspecific divergence than the other GFLV and ArMV RNA2-encoded proteins (Gottula *et al.*, 2013). The 2A<sup>HP</sup>-coding region encodes a higher proportion of non-synonymous to synonymous mutations (Oliver *et al.*, 2010) underlying positive selection that could

reflect virus-host coevolution at a virulence/immunity interface (Jones and Dangl, 2006).

The 2B<sup>MP</sup> movement protein of GFLV forms tubules and functions with the 2C<sup>CP</sup> protein (coat protein) for intercellular virus transport through plasmodesmata (Ritzenthaler *et al.*, 1995). It also interacts with plasmodesmata proteins that interface in a general fashion with other RNA viruses showing similar transport mechanisms (Amari *et al.*, 2010). GFLV is encapsidated by its 2C<sup>CP</sup> protein formed into a 30nm multimeric icosahedral particle with pseudo-T3 symmetry (Lai-Kee-Him *et al.*, 2013). GFLV RNA1, RNA2 and its RNA satellite are separately encapsidated (Quacquarelli *et al.*, 1976; Pinck *et al.*, 1988).

GFLV is rarely seed transmitted (Martelli *et al.*, 2003) and, like most other plant viruses, is a vector specifist (Power, 2008). The longidorid ectoparasitic nematode species *X. index* is primary agent of GFLV transmission (Andret-Link *et al.*, 2004). TRSV and ToRSV are transmitted by *X. americanum sensu lato* and ArMV is transmitted by *X. diversicaudatum*. The nematode-specific basis of nepovirus transmission specificity is uncertain. Different strains of *X. index* reproduce at different rates but do not differ in GFLV transmission competencies (Demangeat *et al.*, 2010). The virus-encoded specificity of transmission has been mapped using reverse genetics approaches. An 11 amino acid region of the coat protein (in the  $\beta$ B- $\beta$ C loop of the two-fold axis of the coat protein junction) determines transmission specificities of *X. index* (Schellenberger *et al.*, 2010) and *X. americanum* (Marmonier *et al.*, 2010).

The host range of nepoviruses varies from narrow or moderate to wide. The

host range of GFLV includes several species of *Chenopodium* and *Nicotiana* (Dias, 1963). *Cucumis sativus* and *Phaseolus vulgaris* were also reported to be experimental hosts of GFLV (Dias, 1963), but these results could not be reproduced (J. Gottula and J. P. Hart, unpublished). Although *Cynodon dactylon* (bermudagrass) was reported to be a host (Izadpanah *et al.*, 2003), the primary host of GFLV in the agroecosystem is grapevine (Andret-Link *et al.*, 2004). The host range of ArMV and ToRSV is wider than GFLV (Ghotbi *et al.*, 2009). Although tobacco is resistant to GFLV, it can efficiently replicate GFLV in tobacco (BY-2) cell cultures (Laporte *et al.*, 2003), suggesting that the basis of resistance is not cell-autonomous.

Mutation rates for RNA viruses have been estimated to be  $1 \times 10^{-5}$  to  $1 \times 10^{-3}$  substitutions per site per round of replication. These high rates have been attributed to the lack of proofreading capabilities of RNA-dependent RNA polymerases (Holmes, 2009), and positive selection for high mutation rates in RNA viruses (Hicks and Duffy, 2011). Iteratively tested sequences in laboratory and field settings have illustrated that GFLV mutations are fixed at a much lower-than-expected frequency (Vigne *et al.*, 2004; Vigne *et al.*, 2013). This can be understood in light of selection, where both protein-coding and non-protein-coding mutations can confer reduced viral fitness (Holmes, 2009).

Haplotype surveys of different GFLV isolates have revealed considerable diversity. Some surveys have focused primarily on GFLV RNA2 2B<sup>MP</sup> (Sokhandan-Bashir and Melcher, 2012) and 2C<sup>CP</sup> sequences (Vigne *et al.*, 2004), but others have expanded the sequencing efforts to include 1E<sup>RdRp</sup> and 2A<sup>HP</sup> sequences (Mekuria *et al.*, 2009; Oliver *et al.*, 2010). Currently there are five fully sequenced GFLV isolates:

F13 from France (Ritzenthaler *et al.*, 1991; Serghini *et al.*, 1990), WAPN172 and WAPN6132 from Washington State (Mekuria *et al.*, 2013), GHu from Hungary (Vigne *et al.*, 2013) and SAPCS3 from South Africa (Lamprecht *et al.*, 2012).

Sequence analyses have revealed GFLV isolates are not unified geographically or by grapevine scion genotype or rootstock. Instead, large swaths of the total scope of GFLV diversity may be present in single fields where *X. index* transmission is occurring (Oliver *et al.*, 2010; Sokhandan-Bashir *et al.*, 2012).

While most of the diversity in GFLV is due to divergence, recombination has played an important role in shaping the population structure. The mechanism of action is thought to be template switching during replication of distinct viral RNAs in a co-infected cell. Numerous GFLV-GFLV recombinants exist (Mekuria *et al.*, 2009; Zarghani *et al.*, 2013) and diverse GFLV-ArMV RNA2 recombinants containing identical breakpoints suggest a hotspot of recombination at the 3' extremities of the 2A<sup>HP</sup>-2B<sup>MP</sup> sequences in GFLV RNA2 (Oliver *et al.*, 2010). For example, GFLV-GHu RNA2 is a recombinant between GFLV and ArMV RNA2 in the 5' UTR and 2A<sup>HP</sup>-2B<sup>MP</sup> coding region (Vigne *et al.*, 2008). No GFLV/ArMV reassortants in the 2C<sup>CP</sup> have been reported, and this gene shows higher interspecific diversity than any other gene surveyed (Gottula *et al.*, 2013).

Multi-partite viral genomes occasionally reassort (Moury *et al.*, 2006). Comparisons of phylogenetic trees of GFLV RNA1 and RNA2 suggest natural reassortants exist, as seen in the alternate phylogenetic groupings of the 1E<sup>RdRp</sup> and 2A<sup>HP</sup> sequences of variant CACSC3 (Oliver *et al.*, 2010). An ancient reassortment event may have played a role in the evolution of GDefV as well (Elbeaino *et al.*,



2012). Large nepovirus satellite RNAs may reassort between virus species or strains (Lamprecht *et al.*, 2013; Chapter 2). Nepovirus reassortment would require coinfection of two nepovirus genotypes in the same cell and nematode uptake of virus particles containing alternate genome parts or satellite RNAs. GFLV reassortants can also be made in the laboratory (Vigne *et al.*, 2013; Chapter 4).

Two types of nepovirus satellite RNAs have been reported including type A and type B satellite RNAs (Fritsch and Mayo, 1993). Type A satellite RNAs are around 200bp, viroid-like and non-protein coding, and type B satellite RNAs are around 1kb, protein coding, and behave like genomic RNAs in terms of replication and encapsidation, except they are dispensable to the helper virus (Mayo, 1991). Very little is known about either type of nepovirus satellite RNA, though basic replication mechanisms of type A satellite RNAs have been described (Roosinck and Sleat, 1992; Etschied *et al.*, 1995).

## ***DISEASE MANAGEMENT***

Resistance is the basis of integrated pest management but sources of resistance are not always available. *Vitis* and *Muscadinia* species are incredibly diverse (Reisch *et al.*, 2011; Myles *et al.*, 2012), but over sixty years of resistance screening have not conclusively produced proven natural GFLV resistance in any grapevine genotype (Oliver and Fuchs, 2011). No sources of resistance have been discovered at least in part to the difficulty in inoculating grapevines (Valat *et al.*, 2003). In the absence of resistance, control measures are accomplished by preventing introduction of the virus and control of nematode vectors.

There is no cure for nepovirus-infected grapevines in vineyard situations. Infected plants can be cured of the virus through tissue culture procedures including meristem tip culture and thermotherapy (Gambino *et al.*, 2009). Although tissue culture is expensive and labor-intensive, this process is a viable procedure to eliminate viruses from infected otherwise valuable grapevine clones (M. Fuchs, personal communication).

In the absence of resistance, the best way to manage a virus disease is to prevent its introduction. This aphorism is especially true for perennial crops such as grapevine. Foundation Plant Services, a unit of the University of California Davis, provides clean, virus-tested certified scion and rootstock materials to US nurseries (Rowhani *et al.*, 2005). Similar grapevine virus testing and certification programs are also underway in Europe and elsewhere (M. Fuchs, personal communication).

Managing nematode vectors can be difficult given the current ban on methyl bromide and other nematicides. In the absence of reliable agrochemicals against nematodes, alternative methods of *X. index*/GFLV control have been explored including fallow periods (Villate *et al.*, 2012), cover crops (Villate *et al.*, 2012), cross protection (Komar *et al.*, 2008) and biocontrol agents (Daragó *et al.*, 2013), each of which is not fully effective and likely not economically attractive. One strategy that has shown promise and is in use commercially is to plant grapevines grafted onto rootstocks that are resistant to *X. index* (Hwang *et al.*, 2010). Research is ongoing to use biotechnology approaches to produce plants with nematode resistance (Li *et al.*, 2011; Yang *et al.*, 2013), but experience with *X. index* resistant rootstocks have shown that resistance to *X. index* is not sufficient for full control of GFLV (Oliver and Fuchs,

2011).

There is currently a critical need for more effective GFLV control measures. Scientific and commercial perspectives agree that the most effective control strategy will likely come from resistance at the rootstock level. Because no resistance to GFLV is found in *Vitis* spp., pathogen-derived resistance or other forms of transgenic resistance could provide a sound basis to impart resistance to GFLV.

### **BIOTECHNOLOGY**

Pathogen-derived resistance to GFLV could provide a means to achieve resistance in grapevine rootstocks. Challenged grapevines grafted onto transgenic rootstocks expressing the GFLV strain F13 coat protein gene in naturally field vineyards showed resistance in three of 16 lines (Vigne *et al.*, 2004), though subsequent tests of these lines in a different field environment challenged with presumably different GFLV strains did not show resistance (M. Fuchs and O. Lemaire, unpublished results). Similarly, transgenic expression of an ArMV CP gene in *V. rupestris* showed no immunity following grafting onto ArMV-infected plants (Spielmann *et al.*, 2000). Transgenic GFLV resistance strategies that involve plantibodies and hairpin RNAs potentially show promise (Andret-Link *et al.*, 2004). Because pathogen-derived resistance has shown efficacy in multiple crops and against diverse viruses, it is plausible that a soundly designed construct could produce effective nepovirus resistance in grapevines.

A GFLV vector (e.g. a virus-induced gene silencing vector) for grapevine functional genomics would be incredibly beneficial for the grapevine research

community (Chapter 3). The ideal GFLV vector will produce reliable systemic infection in grapevine, stably express proteins and silence endogenous genes, and would not result in deleterious effects in inoculated plants or in vineyards in which the vector is introduced.

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## CHAPTER 2

### ***GENETIC VARIABILITY, EVOLUTION AND BIOLOGICAL EFFECTS OF GRAPEVINE FANLEAF VIRUS SATELLITE RNAS***

#### ***ABSTRACT***

Large satellite RNAs (type B satRNAs) of *Grapevine fanleaf virus* (GFLV) from the genus *Nepovirus*, family *Secoviridae* were identified in a naturally infected vineyard and a grapevine germplasm collection. These GFLV satRNA variants had a higher nucleotide sequence identity with satRNAs of *Arabis mosaic virus* (ArMV) strains NW and J86 (93.8 to 94.6%) than with the satRNA of GFLV strain F13 and those of other ArMV strains (68.3 to 75.0%). Phylogenetic analyses showed no distinction of GFLV and ArMV satRNAs with respect to the identity of the helper virus. Seven stretches of 8 to 15 conserved nucleotides (I-VII) were identified in the 5' region of subgroup A nepovirus genomic RNAs (GFLV, ArMV, and *Grapevine deformation virus*) and nepovirus type B satRNAs, including previously reported motif I, suggesting that large satRNAs might have originated from recombination between an ancestral subgroup A nepovirus RNA and an unknown RNA sequence with the 5' region acting as a putative *cis-replication* element. A comparative analysis of two

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GFLV strains carrying or absent of satRNAs showed no discernable effect on virus accumulation and symptom expression in *Chenopodium quinoa*, a systemic herbaceous host. This work sheds light on the origin and biological effects of large satRNAs associated with subgroup A nepoviruses.



## **INTRODUCTION**

*Grapevine fanleaf virus* (GFLV) is the primary causal agent of fanleaf degeneration disease of grapevine. This virus causes severe economic losses worldwide (Andret-Link *et al.*, 2004). *Arabidopsis mosaic virus* (ArMV) and *Grapevine deformation virus* (GDefV) also cause fanleaf degeneration in central Europe (Martelli *et al.*, 2006). GFLV, ArMV and GDefV are subgroup A members of the genus *Nepovirus* in the family *Secoviridae* and have similar bipartite single-stranded RNA genomes (Sanfaçon *et al.*, 2009). GFLV and ArMV are closely related (Andret-Link *et al.*, 2004; Sanfaçon *et al.*, 2009) and GDefV may result from recombination between GFLV and ArMV (Elbeaino *et al.*, 2012, Ghanem-Sabanadzovic *et al.*, 2005).

Nepoviruses, including some GFLV and ArMV isolates, contain large and small satellite RNAs (satRNAs) (Fritsch and Mayo, 1993). Large satRNAs, which tend to be greater than 1 kb and have an open reading frame (ORF), are referred to as type B satRNAs (Mayo, 1991). They are absolutely dependent on a helper genome for replication and encapsidation, and encode a nonstructural protein. Little is known about the origin and function of nepovirus type B satRNAs or their encoded protein. The satRNA associated with GFLV strain F13 is 1,114 nucleotides (nts) long and encodes a 37-kDa protein called P3. This is the only large satRNA characterized so far for GFLV (Fuchs *et al.*, 1989; Hans *et al.*, 1993; Moser *et al.*, 1992; Pinck *et al.*, 1988) although a new GFLV satRNA was recently reported (Lamprecht *et al.*, 2012). SatRNAs of ArMV range from 1,092 to 1,139 nts in size and produce a protein of 39 kDa (Liu *et al.*, 1990; Wetzel *et al.*, 2006). SatRNAs associated with three ArMV isolates from Neustadt an der Weinstrasse (NW) in Germany are 99% similar at the

nucleotide level, while satRNAs associated with other isolates differ greatly, showing as low as 57% amino acid and 73% nucleotide identity to NW (Wetzel *et al.*, 2006). Interestingly, ArMV-NW satRNAs have slightly higher identity to the GFLV-F13 satRNA than to some other ArMV satRNAs (Wetzel *et al.*, 2006). Replication of nepovirus satRNAs by helper viruses is achieved with some degree of specificity. The ArMV satRNA can be replicated by satRNA- deficient ArMV strains Ash and Ivy but not by ArMV strains Hop or AB10 or the genome of other nepoviruses, including GFLV (Liu *et al.*, 1991a). The *Tomato black ring virus* (TBRV) satRNAs replicate only with certain isolates of TBRV, owing specificity to either a helper virus-encoded factor (Fritsch and Mayo, 1993) or the protein encoded by the satRNA (Hemmer *et al.*, 1993, Oncino *et al.*, 1995). In contrast, the satRNA of GFLV-F13 replicates in *Chenopodium quinoa* plants infected with satRNA-deficient GFLV strain TU (Pinck *et al.*, 1988) or ArMV (Fuchs *et al.*, 1991; Hans *et al.*, 1993). SatRNAs are not known to have similarity to sequences available in GenBank, except a short sequence conserved within the 5' end of nepoviruses (Fuchs *et al.*, 1989).

There is no clear association of nepovirus type B satRNAs and viral virulence (Collmer *et al.*, 1992; Roosinck *et al.*, 1992). For example, symptoms of GFLV-infected grapevines do not seem to be influenced by presence or absence of satRNAs (Saldarelli *et al.*, 1993). On model hosts, information on satRNA- induced symptoms is contradictory, with some studies failing to detect an association (Fritsch and Mayo, 1993) and others showing an effect on symptoms and virus accumulation in a host species-dependent manner. For example, the ArMV-lilac satRNA does not promote significant differences in ArMV titer in *C. quinoa*, but prevents virus-induced tip

necrosis (Liu *et al.*, 1991b). An experiment using GFLV strains devoid of satRNAs, to which transcripts of the satRNA of GFLV-F13 were added, suggested a slight delay (1 to 2 days) in symptom development in *C. quinoa* (Fuchs *et al.*, 1991), but this study did not rely on GFLV strains with a homogenous genetic background.

The type B satRNA is fairly prevalent in GFLV or ArMV isolates. Surveys of grapevine collections or virus cultures acquired from geographically diverse origins detected a satRNA in 5 out of 34 GFLV-infected samples by RNA hybridization (Saldarelli *et al.*, 1993), and in 6 of 38 ArMV-infected samples using reverse transcription- polymerase chain reaction (RT-PCR) (Wetzel *et al.*, 2006). No information is available on the occurrence and distribution of type B satRNA in naturally infected commercial vineyards. This study addresses the GFLV satRNA origin, epidemiology, genetic variability, and effect on helper virus multiplication and symptomatology. This aim was to characterize satRNAs in a naturally GFLV-infected vineyard, compare their genetic structure to those of known GFLV and ArMV satRNAs and genomic RNAs, and determine their effect on GFLV virulence on the model host *C. quinoa*.

## ***MATERIALS AND METHODS***

### **Plant material**

Grapevine leaf samples were collected on 14 May 2010 and another set on 10 May 2012 in Lodi, CA. Fifty nine samples were taken from a vineyard that contained a mixture of rootstock genotypes. These vines were established on a site where *Vitis vinifera* ‘Zinfandel’ scions grafted onto Freedom (1613-59 × Dog Ridge) rootstocks

were previously grown (Oliver *et al.*, 2010). Samples were also taken in 2010 from two nearby vineyards, including 16 samples from a vineyard of *V. vinifera* ‘Zinfandel’ scions grafted onto Freedom rootstocks, and eight samples from a vineyard of *V. vinifera* ‘Cabernet Sauvignon’ scions grafted onto Dog Ridge (*V. champini*) rootstocks. Each vineyard was naturally infested with GFLV-viruliferous *Xiphinema index* and ArMV was not present (Oliver *et al.*, 2010). Leaf samples were also collected on 15 June 2010 on GFLV-infected vines at the cold-hardy grape germplasm collection, USDA-Plant Genetic Resource Unit (PGRU), Geneva, NY.

### **GFLV and satRNA detection by ELISA and IC-RT-PCR**

Double-antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) and immunocapture-reverse transcription (IC-RT) polymerase chain reaction (PCR) was conducted with specific GFLV antibodies (Bioreba Inc., Reinach, Switzerland). For ELISA, absorbance at OD405nm was read by a Synergy2 microplate reader and analyzed using Gen5 software (Biotek Corporation, Winooski, VT). The mean absorbance of two in-plate replications for each ELISA sample value was taken. Absorbance values of test samples were blanked by subtracting the absorbance value of a GFLV-free grape leaf or *C. quinoa* leaf extract.

Reverse transcription was conducted following capture of GFLV virions (Vigne *et al.*, 2004) using AMV reverse transcriptase and an 18-mer poly-T primer (New England Biolabs, Ipswich, MA) following manufacturer’s protocol. PCR was conducted on cDNA with GoTaq PCR mixture (Promega, Southampton, UK). The GFLV satRNA was detected using primers P1 and P2, and GFLV RNA2 was detected

using primers P3 and P4 (**Table 2-1**). All DNA amplicons were size fractionated by electrophoresis on a 1.5% agarose gel in a TAE buffer. Positive controls included the satRNA of GFLV strain F13 (Pinck *et al.*, 1988) and isolate R3 from Lodi, CA (this study). Negative controls for PCR included a water control in place of RNA template, and GFLV-free grape or *C. quinoa* cDNA produced from the IC-RT step.

### **5' Rapid amplification of cDNA ends (RACE) and 3' amplification**

A 5' RACE procedure and 3' amplification were carried out to determine the nucleotide sequence of the termini of GFLV satRNA sequences. Immunocapture was obtained from extracts of *C. quinoa* plants infected with satRNA-containing GFLV strains R6-40 and R2-39, as well as satRNA negative GFLV strains R6-18 (this study) and FF, the later being obtained from *in vitro* transcripts of GFLV-F13 RNA1 and RNA2 cDNAs (Viry *et al.*, 1993). Complementary DNA was synthesized as described above with poly-T primers (for 3' amplification) or P2 (for 5' RACE). For 5' RACE, dATP was joined to the 3' ends of cDNAs with terminal deoxynucleotide transferase (New England Biolabs) and products were amplified by PCR first with P5 and P6, and then with P7 (corresponding to the specific sequence in P5) and SP2. Initial 3' amplification was conducted with P5 and P1, followed by a second round of PCR with P1 and P7.

### **Cloning and sequencing PCR amplicons of GFLV genomic RNA and satRNAs**

Size-fractionated PCR products were extracted from gels using an Omega Gel Extraction Kit (Omega Biotek, Doraville, GA), T/A cloned into PCR4-TOPO

**Table 2-1** Oligonucleotides used in the study for GFLV RNA2 or satRNA detection, for 5' RACE and 3' amplification of GFLV satRNAs by IC-RT-PCR

Primer #	Primer name	Sequence 5'-3' <sup>a</sup>
1.	NepSatF	CGTGTAAGCACCGTGCACG
2.	NepSatR	GGCTAATGAGCAACCAAAATCCC
3.	G34	CTWGATTTTAGGCTCAATGGTAT
4.	G37	AAGAAACGAGAACCAATCTCAA
5.	oligo dT-Target	GCTGTCAACGATACGCTACGTAACGGCATGACAGTGT <sub>(18)</sub>
6.	SP1	ACTGCTGTTTGTGTCCAAGCGACACT
7.	TargetF	GCTGTCAACGATACGCTACGTAACGGCATGACAGTG
8.	SP2	GCGGGGCCACAGCAGAAGGACCCTGACCCATT
9.	G38	CTTGCTGGTCAAAGTCAGAG
10.	G39	ATAAATTTGCAAAACAGTAAAAAGA

<sup>a</sup> Numeric subscript 18 indicates the presence of 18 T nucleotides

(Invitrogen, Carlsbad, CA) and transformed into chemically competent TOP10 *E. coli*. Plasmids were extracted from single colony transformants with Plasmid Mini-Prep Kit (Omega) and digested with *EcoRI* (New England Biolabs) to confirm expected size fractions. Sequences were generated via Sanger sequencing with the M13 primer sets at the Cornell University Life Sciences Core Laboratories Center and full bidirectional coverage was obtained for each TOPO-cloned insert.

### **Phylogenetic trees and genetic analyses**

Alignments for GFLV and ArMV genes 1E<sup>Pol</sup>, 2A<sup>HP</sup>, 2B<sup>MP</sup>, 2C<sup>CP</sup>, and satRNAs were created using all full-length or almost full-length sequences available in GenBank. Other genes (1A, 1B<sup>Hel</sup>, 1C<sup>VPg</sup>, and 1D<sup>Pro</sup>) were not considered in this study because of the present paucity of publically available sequence information. SatRNA nucleotide sequences were aligned with genomic RNA 5' UTRs with the Muscle algorithm (Edgar, 2004) in Seaview (Gouy *et al.*, 2010), and these alignments were converted to FASTA files and uploaded to WebLogo3 to summarize sequence conservation (Crooks *et al.*, 2004) of the 5' UTR of satRNAs, genomic GFLV RNAs, genomic ArMV RNAs, and genomic GFLV, ArMV, and GDefV RNAs combined. Base compositions of nepovirus subgroup A genomic RNAs and satRNAs (excluding all gaps) were determined by Seaview and independence of base composition was tested with  $\chi^2$  tests.

Phylogenetic trees were made and sequences statistically analyzed as previously described (Alabi *et al.*, 2011). Briefly, sequences were aligned using ClustalW (Larkin *et al.*, 2007) and manually curated in Seaview to maintain expected

open reading frames (ORFs). The alignments were subjected to the ‘find best nucleotide models’ program option of MEGA5 for maximum likelihood trees, and phylogenetic trees were constructed using 5,000 bootstrap replications using the maximum likelihood method (Tamura *et al.*, 2011). Branches with less than 50% bootstrap support were collapsed. Genetic distance was calculated for each gene within and between virus species in MEGA5 using the maximum composite likelihood model. DnaSP (Librado and Rozas, 2009) was used to conduct Tajima’s neutrality test and to calculate Wright’s  $F_{ST}$ , Hudson’s statistics  $K_{ST}^*$  and  $S_{nn}$  for each gene within and between virus species. DataMonkey software (Kosakovsky Pond and Frost, 2005) set to SLAC default parameters was used to discover all nonsynonymous (dN) and synonymous (dS) mutations and dN/dS ratio from GFLV and ArMV alignments of each gene. Protein masses and isoelectric points were computed from the open reading frame of satRNAs using the Protean software in the Lasergene 9 genetic analysis package (DNASTAR, Madison, WI).

### **Transfer of GFLV isolates carrying satRNAs from grapevine tissue to *C. quinoa***

Frozen GFLV-infected grapevine leaves (R1 through R11) from Lodi, CA, were ground in inoculation buffer (15 mM Na<sub>2</sub>HPO<sub>4</sub>, 35 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.0, and 2% nicotine) and crude extract was pestle-inoculated onto four-leaved *C. quinoa* plants dusted with corundum. Forty-five to fifty-five *C. quinoa* were inoculated per GFLV isolate. Uninoculated apical leaves were tested for systemic infection by ELISA 20 days after inoculation. Plants were characterized for the presence of a satRNA by IC-RT-PCR as described above. The partial RNA2 of GFLV isolate R6



from grape and isolates R6-18 and R6-40 from *C. quinoa* was amplified by IC-RT-PCR and sequenced using primers P3 and P4, and P9 and P10. Each strain used in this study was passaged twice on *C. quinoa* before sequencing or use in experiments.

### **Effects of satRNAs on GFLV multiplication and symptoms in *C. quinoa***

GFLV strains F13 (Vuittenez *et al.*, 1964), FF (Viry *et al.*, 1993), and R6-40 and R6-18 (this study) were used to test biological effects of the satRNA. Strain F13 contains a satRNA (Fuchs *et al.*, 1989; Hans *et al.*, 1993; Pinck *et al.*, 1988) while strain FF does not (Viry *et al.*, 1993). GFLV R6-18 and R6-40 were obtained from passaging infected grapevine tissue of isolate R6 from Lodi, CA to *C. quinoa*. Independent experiments were carried out to test the effect of satRNAs on either virus multiplication or symptom effects. Each experiment was repeated once. All plants were randomized on a greenhouse bench and the identities of each treatment concealed through the course of the experiment. *C. quinoa* were grown to the four leaf stage and mechanically inoculated with crude sap of infected *C. quinoa* leaves as described above. The greenhouse was maintained at 28°C with a 16-h day length. Groups of 20 (virus multiplication) or 10 (symptomatology) plants were inoculated with each isolate. For the virus multiplication experiment, five plants were sampled at four, seven, 13, and 20 days post-inoculation (dpi) and tested by DAS-ELISA. For the symptom experiment, symptoms were rated twice daily for nine days followed by once daily for 14 days, from four to 26 dpi. Six symptom categories were noted including apical leaf curling (category 1), crumpling (category 2), vein clearing (category 3), expanded-leaf flecking (category 4), yellowing/mottling (category 5),

and lateral leaf vein banding (category 6). At 27 dpi, after conclusion of symptom analysis, each plant's above ground fresh weight and height were recorded, and above ground portions were dried in a cool greenhouse for two weeks at which time dry weight was recorded.

### **Statistical analyses of symptom, physiological, and virus titer experiments**

Statistical tests were conducted in SAS (SAS Institute, Cary, NC) for the virus accumulation experiment and the experiment that tested the satRNA effect on symptoms and physiology. Each plant inoculated with a given virus strain was considered a replicate. Each data set was subjected to ANOVA followed by Tukey as a post-hoc test using the GLM procedure in SAS. For the symptom study, data for the six symptom categories was converted into a binary value (1 for presence of the symptom, and 0 for absence of the symptom), and the values were summed for each plant at each time point and this number was considered symptom severity. Area under the disease progress curve (AUDPC) was calculated for symptom severity over time (Jeger and Viljanen-Rollinson, 2001). Virus strains were compared for AUDPC, plant height, plant fresh weight, and plant dry weight.

## ***RESULTS***

### **Grapevine leaf collection and GFLV satRNA detection and sequencing**

Grapevine leaves were collected from 83 vines showing symptom characteristics of GFLV, e.g., foliar mosaic, chlorosis deformation, and shortened internodes, in three naturally infected vineyards in Lodi, CA in 2010 and 2012. The

presence of GFLV was confirmed in symptomatic samples by DAS-ELISA and a satRNA was detected by IC-RT-PCR in GFLV-infected leaf samples from only one of the three vineyards surveyed. The satRNA was scattered throughout this vineyard but, among the 25 five-vine blocks where multiple GFLV-infected leaf samples were taken, nine blocks contained vines where all samples tested positive for the satRNA, seven contained only vines that tested negative for the satRNA, and nine contained vines that tested positive or negative for the satRNA. An unusual angular mosaic symptom was observed in one area of the vineyard containing satRNAs, although typical GFLV symptoms were observed throughout the vineyard, but presence of the satRNA did not correlate to this unusual GFLV symptom given its widespread distribution throughout the field (data not shown).

DNA amplicons of GFLV satRNA obtained by IC-RT-PCR from 11 leaf samples were gel extracted, cloned, and sequenced. SatRNA nucleotide sequences from Lodi, CA showed at least 94% identity with each other, but only up to 78% with the satRNA of GFLV-F13. Additionally, a GFLV isolate from the USDA-Plant Genetic Resource Unit (PGRU) in Geneva, NY, “PGRU accession 106”, had a satRNA with 94 to 98% identity at the nucleotide level with satRNAs from Lodi, CA and 77.5% with the GFLV-F13 satRNA.

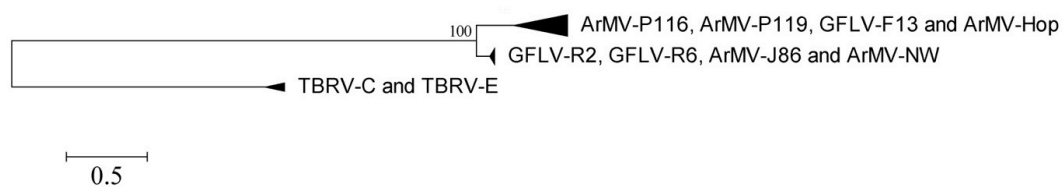
The full-length nucleotide sequence of the satRNA associated with GFLV isolates R6 and R2 from Lodi, CA was determined. They are each 1,140 nts long, compared with 1,114 nts of GFLV- F13 and 1,092 to 1,139 nts of ArMV satRNAs. The GFLV satRNAs from Lodi, CA have a 24-nt 5' UTR, 78-nt 3' UTR, and a single ORF of 1,038 nts. The full-length sequences of the satRNAs associated with GFLV

isolates R2 and R6 were deposited in GenBank as accessions KC162000 and KC161999, respectively.

### Phylogenetic and sequence analysis

A maximum likelihood tree of GFLV and ArMV large satRNAs was constructed using TBRV large satRNAs C and E as outgroups (**Figure 2-1**). The cladogram shows a clustering of the GFLV satRNAs from Lodi, CA (R2 and R6) with satRNAs of ArMV strains J86 and NW. A second clade has satRNAs of GFLV-F13, ArMV-Lilac, -P116, -P119, and -Hop (**Figure 2-1**). SatRNAs associated with GFLV-R2 and -R6, and ArMV-J86 and -NW show less genetic distance overall or relative to each other than members of the other clade. Interestingly, the GFLV-F13 satRNA appears to share more recent ancestry with ArMV-Hop, -Lilac, -P116, and -P119 satRNAs than GFLV-R2 and -R6 satRNAs. Phylogenetic sequence relationships reflect overall sequence similarities and suggest that large satRNAs of subgroup A nepoviruses do not have a defined lineage based on identity of the helper virus.

In order to ascertain functional and evolutionary aspects of the GFLV satRNA, full-length nucleotide sequence alignments of GFLV and ArMV satRNAs and genes 1E<sup>Pol</sup>, 2A<sup>HP</sup>, 2B<sup>MP</sup>, or 2C<sup>CP</sup> were compared. SatRNAs from either GFLV or ArMV show greater intraspecific genetic distance than genes 1E<sup>Pol</sup>, 2A<sup>HP</sup>, 2B<sup>MP</sup>, or 2C<sup>CP</sup> (**Table 2-2**). SatRNAs show comparable interspecific distance with respect to genes 1E<sup>Pol</sup>, 2A<sup>HP</sup>, 2B<sup>MP</sup>, but less than 2C<sup>CP</sup> which is *sensu stricto*, the gene that defines GFLV/ArMV speciation (Elbeaino *et al.*, 2012). By using Tajima's D as a measure of the frequency and distribution of sequence polymorphisms in a gene to infer its



**Figure 2-1** Phylogenetic relationships of full-length nucleotide sequences of type B satRNAs of *Grapevine fanleaf virus* (GFLV) and *Arabis mosaic virus* (ArMV). Tree was created using a maximum likelihood model. Numbers at the branch junctions represent the percent of trees out of 5,000 replications in which associated taxa are clustered, and the key and corresponding branch lengths are proportionally related to number of substitutions per site. Outgroup is comprised of satRNAs associated with *Tomato back ring virus* (TBRV) isolates C and E blocked together. GenBank accession numbers of the nucleotide sequences used to build the phylogenetic tree are shown in **Figure 2-1** and as follows: ArMV-Hop satRNA, TBRV-C satRNA (X05689) and TBRV-E satRNA (X05687).

microevolutionary history, no significant nonrandom distribution of sequence polymorphisms was obtained for the satRNAs, indicating that the sequence composition cannot be attributed to population expansion or decline. Tajima's D was significantly large for gene 2B<sup>MP</sup>, which could suggest population expansion or balancing selection within ArMV and GFLV populations, although this result is likely attributable to the prevalence of interspecies GFLV/ArMV recombinants within this gene (Sokhandan-Bashir and Melcher, 2012).

In order to better understand the interspecific diversity of satRNAs, sequence alignments were subjected to statistical tests for comparative relationships (**Table 2-3**).  $K_{ST}^*$ , which compares within-group diversity to total diversity (Hudson *et al.*, 1992), signaled genes 2B<sup>MP</sup> and 2C<sup>CP</sup> possess significant species subdivision, whereas genes 1E<sup>Pol</sup> and 2A<sup>HP</sup> and the satRNA do not. The nearest-neighbor sequence statistic ( $S_{nn}$ ) (Hudson, 2000) showed that while genes 1E<sup>Pol</sup>, 2A<sup>HP</sup>, 2B<sup>MP</sup>, and 2C<sup>CP</sup> are highly likely to have their most closely related sequences from the same virus species, the satRNAs nearest neighbors are less likely to be from the same virus species (**Table 2-3**). The satRNA  $F_{ST}$  showed that only 4% of genetic variation is attributable to whether it belongs to GFLV or ArMV, whereas other genes showed 36 to 63% of their variation arising from their GFLV or ArMV identities. All together, three measures of intra-versus interpopulation diversity showed the GFLV and ArMV satRNAs are not differentiated based on the identity of their helper virus, which suggests no species-specific identity for these satRNAs.

The ORFs of the GFLV satRNA from Lodi, CA correspond to a translation product of 346 amino acids, compared with 341 amino acids for GFLV-F13 satRNA

(Hans *et al.*, 1993), and 338 to 360 amino acids for ArMV satRNAs (Wetzel *et al.*, 2006). The GFLV-R2 and -R6 satRNA-encoded proteins have a predicted molecular weight of 37kDa and an isoelectric point of 10.4, near the median of predictions for other GFLV and ArMV satRNA-encoded peptides. The ratio of non-synonymous to synonymous nucleotide substitutions is higher for the satRNA-encoded proteins than for GFLV and ArMV proteins 1E<sup>Pol</sup>, 2A<sup>HP</sup>, 2B<sup>MP</sup>, or 2C<sup>CP</sup> (**Table 2-3**), indicating the satRNA undergoes less negative selection than gene counterparts in its helper viruses. Twenty sites within the satRNA ORF were identified as having significant ( $P < 0.05$ ) evidence for negative selection and none of the sites in the satRNA ORF showed significant ( $P < 0.05$ ) evidence for positive selection. The fact that a large percentage of satRNA protein failed to be identified as under selection, relative to genes present on genomic RNAs, reflects the need for more satRNA sequences to gain a clearer picture of which sites are under negative or positive selection.

### **Relationships of 5'-terminal nucleotide sequences of GFLV and ArMV satellite and genomic RNAs**

Close inspection of the 5' UTR of the GFLV and ArMV satRNAs showed that they possess greater identity to their helper viruses' genomic RNAs than previously realized. While it has been previously noted that the first nucleotides of the GFLV-F13 satRNA were nearly identical to those of nepovirus genomic RNAs (Fuchs *et al.*, 1991; Hans *et al.*, 1993), it is apparent that the first 148 to 155 nts of the GFLV and ArMV satRNAs show significant identity to subgroup A nepovirus genomic RNAs' 5' UTRs (**Figure 2-2**). The conservation is distributed across the first 137 to 191

nucleotides of GFLV, ArMV, or GDefV RNA1 or RNA2. The homologous area ends approximately at the uracil-rich tract in the genomic RNAs (nt 225 in **Figure 2-2**). Much of the homologous sequence is contained within the ORF of the satRNAs (nts 15 to 25 and beyond) and shows six highly conserved stretches (motifs II-VII) of 8 to 15 nts in addition to the first stretch of 12 conserved nucleotides (motif I) previously described (9,13) (**Figure 2-2**). The base compositions of the satRNAs and genomic RNAs are not significantly different before nt 226 in the alignment (**Figure 2-2**) ( $P = 0.471$ ) but are different after this nucleotide position ( $P = 0.0014$ ) according to  $\chi^2$  analysis.

### **Passaging GFLV herbaceous hosts and curing the satRNA**

Ten GFLV-infected grapevine leaf samples from Lodi, CA that contained a satRNA were used to inoculate *C. quinoa*. Infected *C. quinoa* isolates were produced from 6 of the 10 GFLV inocula, as shown by DAS-ELISA and characteristic vein clearing in apical leaves. Multiplex RT-PCR for GFLV RNA2 and the satRNA was performed using total RNA extracted from 10 infected *C. quinoa*. Inoculation with R6 resulted in two infected *C. quinoa* that differed in presence or absence of satRNA: R6-40 contained a satRNA while R6-18 did not. After two additional passages through *C. quinoa*, RT-PCR analysis confirmed presence or absence of the satRNA was maintained (**Figure 2-3**). A partial GFLV RNA2 sequence was obtained for strains R6-18 and R6-40 in infected *C. quinoa* tissue and isolate R6 in grape tissue (GenBank accession number KC162001). The sequences, which include most of gene 2B<sup>MP</sup>, all of gene 2C<sup>CP</sup>, and most of the 3' UTR, are 95% identical to GFLV isolate CACSB3 (GenBank accession number GU972578), which is from the same vineyard in Lodi



**Table 2-2** Genetic parameters of genes 1E<sup>Pol</sup>, 2A<sup>HP</sup>, 2B<sup>MP</sup>, 2C<sup>CP</sup> and the satRNA within and between *Grapevine fanleaf virus* (GFLV) and *Arabidopsis mosaic virus* (ArMV) isolates.

**A. Genetic distance and test of neutrality within and between groups<sup>a</sup>**

<i>Gene</i>	GFLV		ArMV		ArMV vs. GFLV	
	<i>d</i>	<i>Tajima's D</i>	<i>d</i>	<i>Tajima's D</i>	<i>d</i>	<i>Tajima's D</i>
1E <sup>Pol</sup>	0.043	-0.470 ns	0.092	n/a (n=2)	0.181	-0.914 ns
2A <sup>HP</sup>	0.056	-1.170 ns	0.134	-1 ns	0.203	-1.085 ns
2B <sup>MP</sup>	0.053	2.75*	0.107	2.36*	0.201	2.669*
2C <sup>CP</sup>	0.045	-0.741 ns	0.099	-0.954 ns	0.422	-1.0245 ns
SatRNA	0.167	n/a (n=3)	0.256	-0.55 ns	0.212	-0.57 ns

**B. Comparative genetic parameters between GFLV and ArMV populations<sup>b</sup>**

<i>Gene</i>	<i>K<sub>st</sub></i> <sup>*</sup>	<i>P value</i>	<i>S<sub>m</sub></i>	<i>F<sub>st</sub></i>
1E <sup>Pol</sup>	0.0589	0.3821 ns	1	0.53509
2A <sup>HP</sup>	0.0671	0.3950 ns	0.98958	0.44919
2B <sup>MP</sup>	0.02868	0.0000***	0.98653	0.36131
2C <sup>CP</sup>	0.02073	0.0271*	1	0.62785
Satellite	0.00224	0.3423 ns	0.77778	0.04177

<sup>a</sup> Genetic distance and Tajima's D are shown for genes among GFLV or ArMV isolates, and between GFLV and ArMV isolates.

Asterisks represent statistical significance ( $P < 0.05$ ) and "ns" represents lack of statistical significance according to DnaSP algorithm; n/a, not applicable because of small sample size (n).

<sup>b</sup>  $K_{st}$ <sup>\*</sup> and associated  $P$  value,  $S_m$  and  $F_{st}$  statistics are shown for each gene between GFLV and ArMV isolate populations.

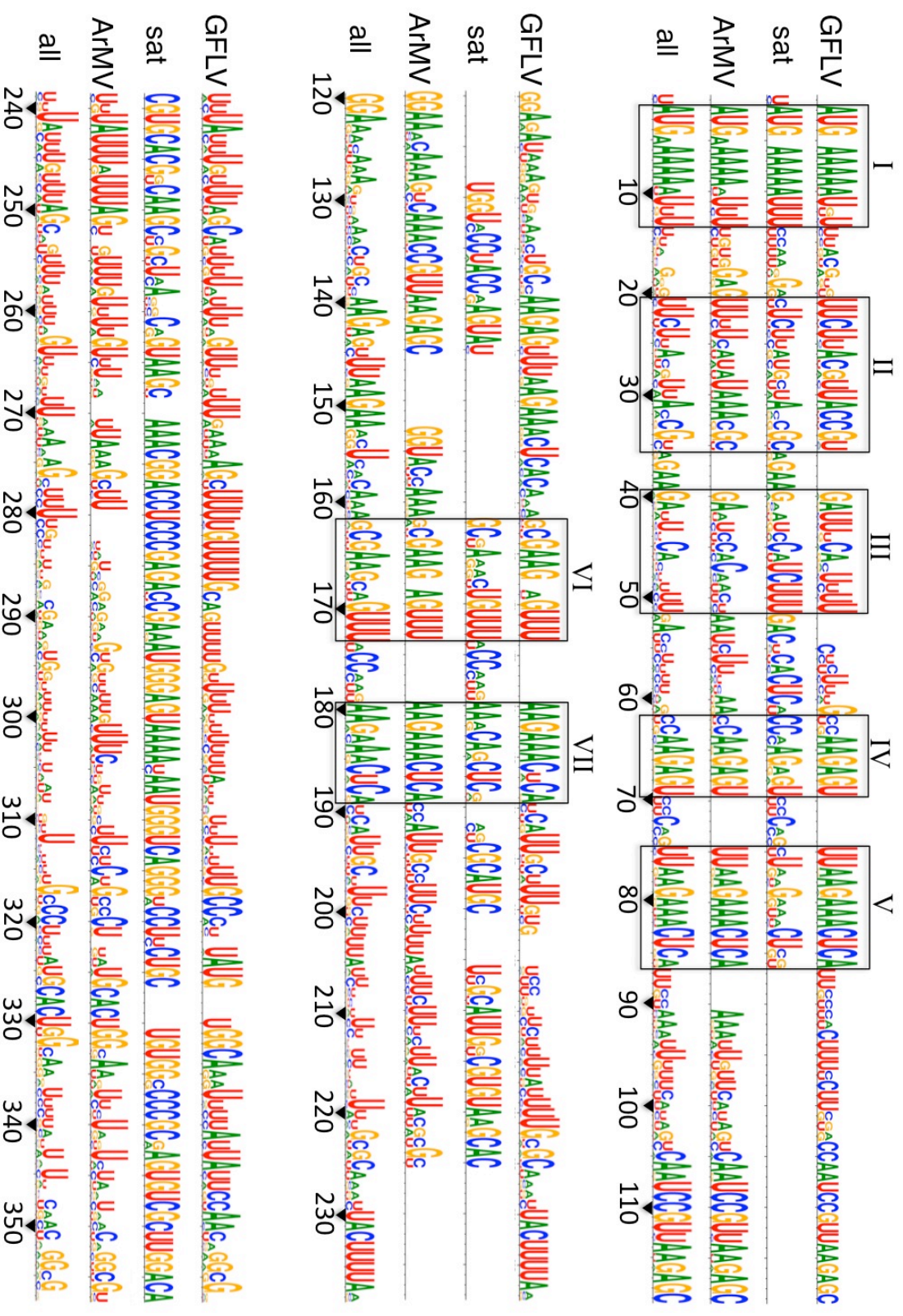
<sup>c</sup> Statistical significance is represented by one ( $P < 0.05$ ) or three ( $P < 0.001$ ) asterisks.

**Table 2-3** Estimates of selection pressures on select subgroup A nepovirus proteins<sup>a</sup>

Gene	Log (L)	Mean	# Positive	% Positive	# Negative	% Negative	# Neutral	% Neutral
1E <sup>Pol</sup>	-12776.9	0.134241	0	0	228	27.67	596	72.33
2A <sup>HP</sup>	-16684.9	0.291038	5	1.76	138	48.59	141	49.65
2B <sup>MP</sup>	-16012.7	0.0430145	0	0	300	84.27	56	15.73
2C <sup>CP</sup>	-39521.8	0.078302	2	0.396	437	86.53	66	13.07
satellite <sup>P3</sup>	-5227.99	0.519967	0	0	20	6.15	305	93.85

<sup>a</sup> Normalized dN/dS ratios (mean) for each gene of GFLV and ArMV and log likelihood (L) scores for fitted models. Number and percent of sites under positive, negative and neutral selection (P<0.05) are noted.

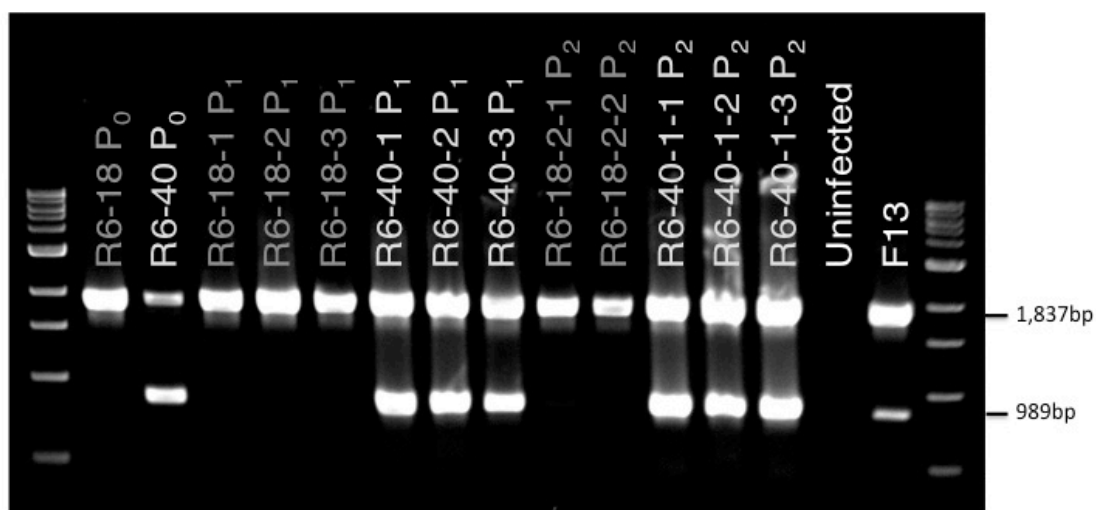
**Figure 2-2** Shared identity of nepovirus subgroup A type B satellite with Nepovirus genomic RNAs. WebLogo graphical display of consensus nucleotide sequence alignments at the 5' region of genomic RNAs from *Grapevine fanleaf virus* (GFLV), nepovirus subgroup A large satellite RNAs (sat), *Arabidopsis mosaic virus* (ArMV), and GFLV, satRNAs, ArMV and *Grapevine deformation virus* (GDefV) (all). Three blocks represent a sequence continuum, and boxed regions within blocks represent conserved nucleotide sequences among genomic and satRNAs. GenBank accession numbers of the nucleotide sequences used in the alignment are as follows: ArMV-P116 satRNA (DQ187318), ArMV-lilac satRNA (D00664), GFLV-F13 satRNA (D00442), GFLV-R2 satRNA (KC162000), GFLV-R6 satRNA (KC161999), ArMV-NW satRNA (DQ187317), ArMV-J86 satRNA (DQ187316), ArMV-P119 satRNA (DQ187319), ArMV-Lv RNA1 (EU617326), ArMV-ba RNA1I (GQ369526), ArMV-ba RNA1II (GQ369527), ArMV-ba RNA1III (GQ369528), ArMV-NW RNA1 (AY303786), GDefV RNA1 (NC\_017939), GFLV-F13 RNA1 (D00915), GFLV-WAPN6132 RNA1 (GQ332373), GFLV-GFV1050-02 RNA1 (JX513889), GFLV-SAPSC3 RNA1 (JF968120), GFLV-WAPN173 RNA1 (GQ332372), ArMV-Lv RNA2 (EU617327), ArMV-ba RNA2IV (GQ369529), ArMV-ba RNA2V (GQ369530), ArMV-NW RNA2 (AY017339), ArMV-S RNA2U (X81814), ArMV-S RNA2L (X81815), ArMV-Ta RNA2 (EF426853), GDefV RNA2 (AY291208), GFLV-WAPN173 RNA2 (GQ332368), GFLV-NW RNA2 (AY027338), GFLV-SAPCS3 RNA2 (JF968121), GFLV-WAPN8133 RNA2 (GQ332369), GFLV-CACSB5 RNA2 (GU972580), GFLV-CACSC1 RNA2 (GU972581), GFLV-CAZINA4 RNA2 (GU972574), GFLV-F13 RNA2 (D00915), GFLV-GHu RNA2 (EF426852), GFLV-WACF2142 RNA2 (GQ332371), GFLV-WACH911 RNA2 (GQ332364), GFLV-WAPN57 RNA2 (GQ332367), GFLV-WAPN165 RNA2 (GQ332365), and GFLV-WAPN1492 RNA2 (GQ332370).



CA (Oliver et al., 2011). The GFLV-R6-18 and GFLV-R6-40 RNA2 sequences were 99.6% identical at the nucleotide level, which showed that the two strains established on *C. quinoa* were nearly identical except for the presence or absence of the satRNA, and these strains also showed similar identity to GFLV from the grapevine source from which they originated. Similarly, cDNAs of viral RNA progeny derived from F13 transcripts show 99.8% nucleotide identity to the wild-type GFLV-F13. Thus, two sets of GFLV sister strains (R6-40 and R6-18, and F13 and FF) differing in presence or absence of satRNAs were created and these were used to characterize the biological effect of the satRNA.

#### **Effect of the satRNA on GFLV accumulation and symptoms in *C. quinoa***

The GFLV-F13 and GFLV-R6 satRNAs were used to determine whether a satRNA has a measurable impact on its helper virus or on the systemic host *C. quinoa*. Viral protein accumulation was measured by DAS-ELISA at four, seven, 13, and 20 dpi (**Figure 2-4**). The effect of time point was significant ( $F = 105.03$ ,  $P < 0.0001$ ), but effect of the virus strain was not ( $F = 1.30$ ,  $P = 0.2825$ ), nor was the interaction of virus strain and time point ( $F = 0.76$ ,  $P = 0.6559$ ). GFLV capsid accumulation increased successively at four, seven and 13 dpi, and these differences were statistically significant ( $P < 0.05$ ). Virus accumulation at 20 dpi was not significantly different from 13 dpi but was significantly different from all other time points. Presence or absence of the satRNA did not significantly impact viral protein accumulation overall or at any time point. This indicates the GFLV satRNA did not



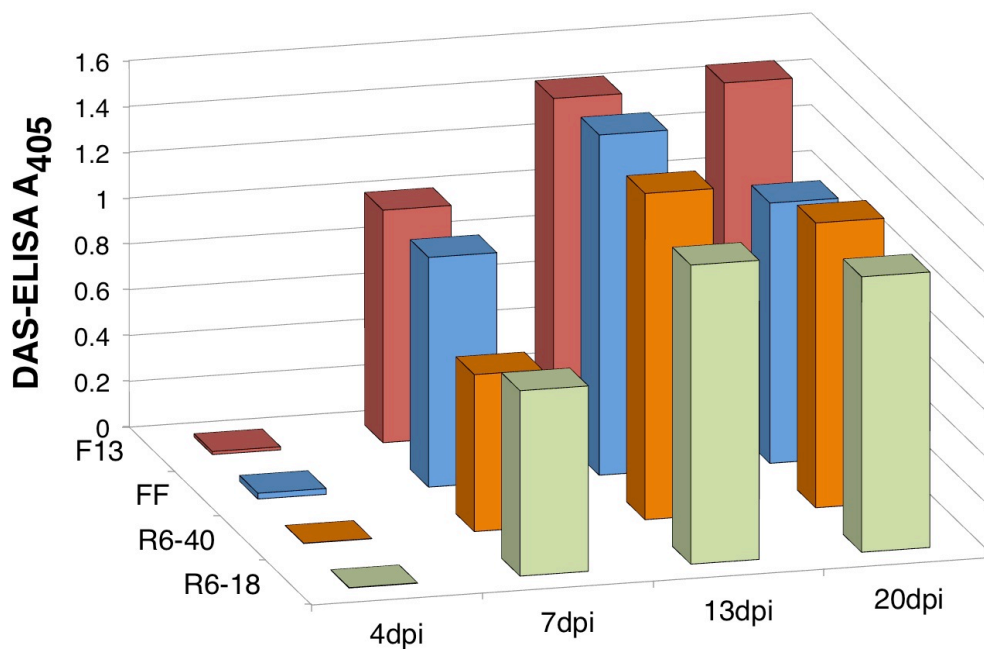
**Figure 2-3** *Grapevine fanleaf virus* (GFLV) strain R6 cured of its satRNA. Duplex IC-RT-PCR for RNA2 and satellite used total RNA extracted from *Chenopodium quinoa* subjected to three consecutive passages (P<sub>0</sub>, P<sub>1</sub> and P<sub>2</sub>). P<sub>0</sub> denotes the initial passage from grapevine to *C. quinoa* plants 18 and 40, and P<sub>1</sub> and P<sub>2</sub> denotes respective serial passages made from crude sap of initially infected plants. The RNA2 DNA amplicon is 1,837bp and the satRNA amplicon is 989bp. GFLV-F13 was used as the positive control for RNA2 and satRNA, and total RNA from a healthy *C. quinoa* constitutes the negative control. DNA fragments were resolved by electrophoresis on 1.5% agarose gels, and a 1kb ladder (New England Biolabs) was used as a marker.

discernably change the competitive host–virus interaction.

The four GFLV strains were inoculated to *C. quinoa* and symptoms were recorded at multiple time points after which plant physiological measurements were taken. Strains produced significant differences for each measurement including symptom severity over time (AUDPC) ( $F = 31.07$ ,  $P < 0.0001$ ), height ( $F = 4.68$ ,  $P = 0.0073$ ), above-ground fresh weight ( $F = 7.93$ ,  $P = 0.0003$ ), and above-ground dry weight ( $F = 3.88$ ,  $P = 0.0168$ ). The GFLV genomic RNAs had a striking impact on symptoms (**Figure 2-5**), though not necessarily on whole-plant physiology (**Figure 2-6**), and the satRNA had no striking impact on either. No significant differences in AUDPC were detected between GFLV strains FF and F13, or between GFLV strains R6-18 and R6-40, but each strain of the F13 background was significantly different from each strain of the R6 background (**Figure 2-5**). This author detected significant differences between GFLV strains for three plant physiological parameters including height, dry weight and fresh weight. The only statistically significant physiological difference observed between sister strains was that fresh weight of FF was higher than that of F13, as well as R6-18 and R6-40 (**Figure 2-6**). Except that FF- infected plants produced higher fresh weight than F13-infected plants, satRNAs did not have a significant measurable impact on *C. quinoa* symptoms or plant physiology.

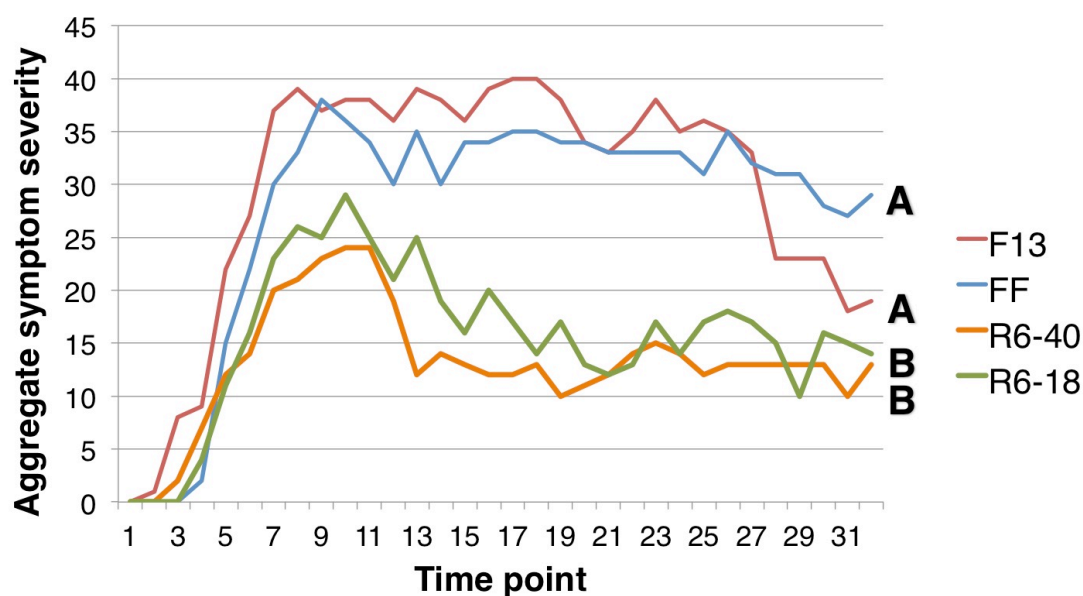
## **DISCUSSION**

GFLV satRNAs that are genetically similar were characterized by IC-RT-PCR and sequencing from vines in a naturally infected vineyard in Lodi, CA, as well as a vine in a USDA-PGRU grape germplasm collection in Geneva, NY. Based on the

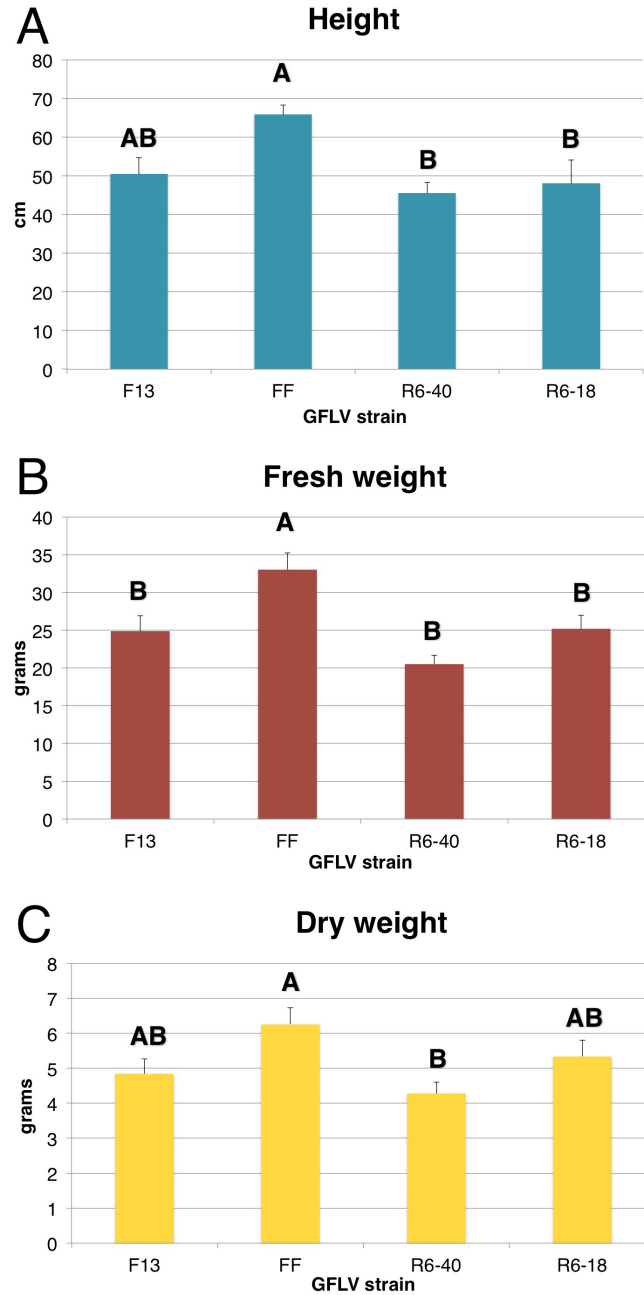


**Figure 2-4** Accumulation of *Grapevine fanleaf virus* (GFLV) capsid in mechanically inoculated *Chenopodium quinoa*. GFLV capsid was measured by double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) at an absorbance of 405 nm, and means of five samples blanked are shown. GFLV strains F13 (red bars – with satRNA), FF (blue bars – without satRNA), R6-40 (orange bars – with satRNA) and R6-18 (green bars – without satRNA) were measured at four, seven, 13 and 20 days post inoculation (dpi). Significant differences for virus accumulation were detected between all time points except between 13 and 20 dpi. Differences in virus protein quantity based on virus strain or virus strain by time point were not significant in ANOVA tests.





**Figure 2-5** Symptom severity over time of *Grapevine fanleaf virus* (GFLV) strains differing in genome and satRNA. Sum symptom severity on *Chenopodium quinoa* of GFLV strains F13 (with satRNA), FF (without satRNA), R6-40 (with satRNA) and R6-18 (without satRNA) (n=10) according to a six category rating scale, shown for 32 time points beginning at four days post inoculation. The first 18 time points represent twice daily ratings, and time points 19 through 34 represent once daily ratings. The height of the line graph represents the sum of all symptom categories among all ten replicates for each virus strain. Area under the disease progress curve (AUDPC) corresponds to the total area under each line. The absence of significant difference according to Tukey ( $P<0.05$ ) is indicated with the same letters.



**Figure 2-6** Physiological parameters of *Chenopodium quinoa* infected with *Grapevine fanleaf virus* strains F13 (with satRNA), FF (without satRNA), R6-40 (with satRNA) and R6-18 (without satRNA) at 34-days-post-inoculation. Significant differences in height (A), fresh weight (B), and above-ground dry weight (C) according to Tukey ( $P < 0.05$ ) are represented with different letters within each graph. Columns represent the mean of 10 replicates and error bars represent standard error.

discovery of a very similar satRNA from disparate sources, records related to the USDA-PGRU accession were searched to attempt to trace the natural history of this satRNA. The GFLV- and satRNA-containing vine at USDA-PGRU (accession 106) is a selection of ‘Grande Glabre’, which was introduced to the United States from the Germplasm Repository in Bordeaux, France in 1962, kept at the USDA-Animal Plant Health Inspection Service Plant Pest Quarantine station in Beltsville, MD, for 5 years prior to introduction to the repository in Geneva, NY. Based on these records, PGRU accession 106 was likely infected at the time of introduction because GFLV and *Xiphinema index*, its ectoparasitic nematode vector (Andret-Link *et al.*, 2004), do not naturally occur in the eastern United States, including New York and Maryland, and pollen-transmission of GFLV is not known. The nucleotide sequence similarities suggest the satRNAs in Lodi, CA may share very recent ancestry with the satRNA that can be traced back to Bordeaux, France in the early 1960s.

Field and laboratory observations support the hypothesis that the helper virus may easily dispense of its satRNA during plant-to-plant transmission. The distribution of the satRNA among GFLV-infected vines in the naturally infected vineyard in Lodi, CA showed that the satRNA was absent from some GFLV-infected plants adjacent to others that contain the satRNA. Similarly, mechanical inoculation of crude sap from GFLV-infected grape leaves containing the satRNA resulted in a GFLV-infected plant that did not contain a satRNA (R6-18). This information suggests that plant-to-plant transmission in field and laboratory settings can result in loss of the satRNA. Conversely, detection of a satRNA in PGRU-106, which could only have been infected along with GFLV over 50 years prior, shows the satRNA can be stably

maintained like its helper virus.

This author did not detect an association between GFLV symptoms in a vineyard with presence of the satRNA. Saldarelli *et al.* (1993) also reported no association between the satRNA and GFLV symptoms in diverse grapevine cultivars. Neither my study, nor Saldarelli *et al.* (1993) evaluated the effect of the satRNA on symptoms and virus accumulation in a common grapevine cultivar. This author tested for satRNA-induced symptom effects in a systemic host of GFLV, *C. quinoa*, but could not discern much impact of two diverse GFLV satRNAs on virus accumulation or symptoms. This was in contrast to a previous experiment showing the association of the ArMV-Lilac satRNA with an amelioration of symptoms in *C. quinoa* (Liu *et al.*, 1991b). Symptom measurements on *C. quinoa* showed greater differences between the two GFLV sister strains selected for this study than differences within GFLV sister strains differing in presence or absence of diverse satRNAs. Together, my data suggests the satRNA has a commensal relationship with GFLV and *C. quinoa*.

The origins of satRNAs are generally not known, but they may originate by chance recombination of viral and/or host nucleic acids (Simon *et al.*, 2004). The type B satRNAs associated with subgroup A nepoviruses have substantial identity with genomic RNAs of the helper virus in their first 150 nucleotides. Based on this extended similarity, a recombination event likely occurred between the 5' UTR of an ancestral subgroup A nepovirus RNA and another unidentified RNA with a putative break point at nucleotide 150. Given that previous analyses of *in vitro* and *in vivo* translation products have demonstrated expression of the full-length satRNA-encoded protein (Hans *et al.*, 1992; Liu and Cooper, 1993; Moser *et al.*, 1992), the

recombination event appears to have given rise to satRNAs encoding a protein composed of an amino terminal region for which the corresponding nucleotide sequence is of a helper virus's 5' UTR. Because of an extended sequence relationship of this 5' satRNA region specifically with ArMV, GFLV or GDefV, and because of the promiscuity of these satRNA associations, it appears satRNAs might have arisen in a common ancestor of these virus species and diversified in a non-helper virus specific fashion. Currently, there is no nucleotide or amino acid sequence available in GenBank that shows continuous identity to the rest of the satRNA sequence beyond the first 150 nucleotides; therefore, the other parental RNA sequence remains unknown.

The evidence that satRNAs have diversified in a helper virus independent manner suggests certain conserved elements of the satRNA must be requisite for functionality in both GFLV and ArMV. Although the protein encoded by the satRNA is indispensable for satRNA replication by a GFLV or an ArMV helper (Hans *et al.*, 1993; Liu and Cooper, 1993), the satRNA protein apparently undergoes less negative selection than other nepovirus genes. The conservation of nepovirus satRNAs and genomic RNAs 5' ends well within the satRNA ORF suggests RNA replication determinants could be maintained within the satRNA 5' UTR and 5' end of its ORF. Like nepovirus satRNAs, the satellite RNA of *Bamboo mosaic virus* (satBaMV) possesses similarity to its helper virus in the 5' untranslated region (Lin and Hsu, 1994). This similarity is biologically significant since the BaMV RdRp specifically interacts with *cis-acting* elements in the satBaMV to initiate replication (Huang *et al.*, 2010), and mutagenic analyses suggest this could also be true for nepovirus satRNAs

(Hans *et al.*, 1993; Hemmer *et al.*, 1993; Liu *et al.*, 1991a).

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## CHAPTER 3

### ***A VIRAL VECTOR COMPOSED OF GRAPEVINE FANLEAF VIRUS***

#### ***ABSTRACT***

Versatile and highly effective tools would be useful for *Vitis* spp. (grapevine) functional genomics. To this aim, a viral vector capable of stable heterologous protein expression and gene silencing was developed from *Grapevine fanleaf virus* (GFLV), a member of the genus *Nepovirus*, family *Secoviridae*. This virus has a bipartite RNA genome, accumulates to high levels in grape berries, and has no recognized tissue tropism. The GFLV vector is from two attenuated, wild type strains or assortants derived thereof and is deficient in transmission by the ectoparasitic nematode *Xiphinema index* because of targeted coat protein mutations. The GFLV vector can be modified by insertion of heterologous genetic material and delivered to plants via agroinfiltration. The GFLV vector exhibits stable expression of Red fluorescent protein following four serial passages and gene silencing capabilities equivalent to *Tobacco rattle virus*, a standard viral vector in herbaceous hosts. The GFLV vector can conduct spatially phased expression of enhanced Green fluorescent protein and Red fluorescent protein when tagged to each of its genomic RNAs. The versatility, effectiveness and biosafety of this grapevine viral vector make it a good candidate platform for grapevine functional genomics research.

## **INTRODUCTION**

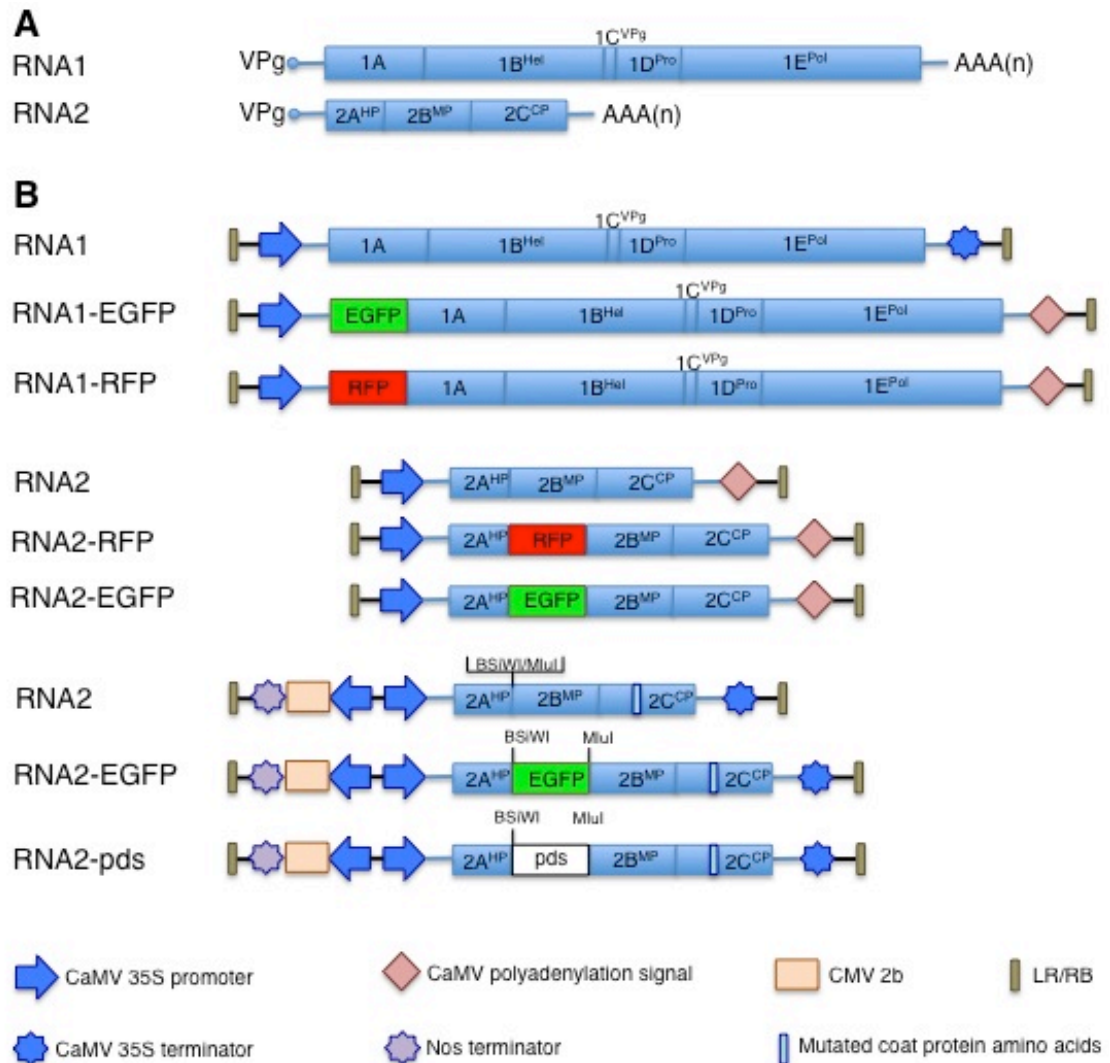
Viral vectors have emerged as key tools for functional genomics in plant biology. Application of viral vectors is a popular approach to validate activity of candidate genes without resorting to cumbersome and time-consuming stable transformation procedures (Vaghchhipawala *et al.*, 2010; Senthil-Kumar and Mysore, 2011). Despite their promise, viral vectors sometimes have limitations such as the quantity of foreign genetic material they can stably support (Arazi *et al.*, 2001; Avesani *et al.*, 2007), a lack of systemic infectivity (Liu *et al.*, 2009) or lack a seamless one-step inoculation method (Satyanarayana *et al.*, 2001, Yoon *et al.*, 2011; Agüero *et al.*, 2012). Furthermore, only few viral vectors have been extensively validated in woody crops (Dawson and Folimonova, 2013).

*Vitis vinifera* is one of the most ancient and most valuable horticultural crops (Myles *et al.*, 2011; Reisch, 2011). Coupling the availability of genome sequences (Jaillon *et al.*, 2007; Velasco *et al.*, 2007; Adam-Blondon *et al.*, 2011) with numerous phenotypes such as berry flavor and stress resistance, there is growing interest in grapevine functional genomics. A lack of rapid and scalable reverse genetics tools hinders functional grapevine gene characterization. Existing platforms for grapevine functional gene characterization encompass (i) stable transformation, which takes eighteen months to three years and is difficult to master (Iocco *et al.*, 2001), (ii) ‘hairy root’ (*Agrobacterium rhizogenes*) transformation, which is in grapevine is prone to intra-plant expression variability and restricted to roots (Yang *et al.*, 2013), and (iii) transient agroinfiltration assays, which can be cultivar specific and inappropriate for some tissues such as roots or grape berries (e.g. Santos-Rosa *et al.*, 2008; Bertazzon *et*

*al.*, 2011; Visser *et al.*, 2012). Given the limitations in grapevine functional genomics platforms, very few grapevine genes have been functionally characterized *in planta* (Martínez-Zapater *et al.*, 2011; Romieu *et al.*, 2011; Tillett *et al.*, 2011). Instead, researchers have generally relied on herbaceous substitutes and *in vitro* biochemical approaches to elucidate the function of candidate genes (Battilana *et al.*, 2011; Mejía *et al.*, 2013; Nicolas *et al.*, 2013). A robust functional genomics platform for grapevine would greatly enhance existing research tools and facilitate improvement of this high value specialty crop.

A handful of viral vectors have been developed for economically important woody plants, including stone fruits, citrus and grapevine (Dawson and Folimonova, 2013). For grapevine, *Grapevine virus A* (GVA) (Muruganantham *et al.* 2009), *Grapevine rupestris stem pitting-associated virus* (GRSPaV) (Meng *et al.*, 2013) and *Grapevine leafroll-associated virus 2* (GLRaV-2) (Kurth *et al.*, 2012) have been engineered to be viral vectors. GVA, GRSPaV and GLRaV-2 have monopartite RNA genomes and are restricted to phloem tissue. In addition, GVA and GRSPaV vectors lack stability or infectivity (Dawson and Folimonova, 2013; Meng *et al.*, 2013).

*Grapevine fanleaf virus* (GFLV) is one of the 64 recognized viruses of grapevine (Andret-Link *et al.*, 2004, Martelli, 2014) In contrast to GVA, GRSPaV and GLRaV-2, GFLV is not phloem restricted and has a bipartite RNA genome (**Figure 3-1A**). RNA1 codes for proteins involved in replication and polyprotein maturation, and RNA2 codes for proteins involved in RNA2 replication, virus movement and RNA encapsidation (**Figure 3-1A**). Both genomic RNA species are necessary for systemic infection (Andret-Link *et al.*, 2004). Here, we report the engineering of GFLV into a



**Figure 3-1** Schematic illustration of the *Grapevine fanleaf virus* (GFLV) genome and vectors. A) GFLV is composed of RNA1 and RNA2 with a 5' genome-linked protein and a 3' poly-A tail. Polyproteins (boxes) are cleaved by the GFLV proteinase (1D<sup>Pro</sup>) into individual protein components (annotated). The polyprotein intervenes 5' and 3' untranslated region (UTRs) represented as horizontal lines. B) GFLV cDNAs were placed downstream of a *Cauliflower mosaic virus* (CaMV) 35S promoter and into binary vectors. Terminal sequences to the GFLV cDNA include a CaMV 35S terminator (RNA1, RNA2, RNA2-EGFP and RNA2-pds) or a CaMV polyadenylation signal (RNA1-EGFP, RNA1-RFP and RNA2-RFP). Sequences of *enhanced Green fluorescent protein* (EGFP), *Red fluorescent protein* (RFP) or a partial *Phytoene*



*desaturase* (pds) from *Nicotiana benthamiana* were inserted within the GFLV cDNAs of RNA1 or RNA2 as shown. Right border (RB) and left border (LB) components of the binary vector are shown. RNA2, RNA2-EGFP and RNA2-pds contain an expression cassette for the 2b silencing suppressor of *Cucumber mosaic virus*. The vector component derived from RNA2 was modified to contain a multiple cloning site (MCS) composed of unique BsiWI and MluI restriction sites, a synthetic polyprotein cleavage site intervening the MCS and 2B<sup>MP</sup> and a mutation in the coat protein coding region for abolition of transmissibility by the ectoparasitic nematode *Xiphinema index*, except for RNA2-RFP. Heterologous sequences were inserted into RNA1 or RNA2 as shown.

viral vector for grapevine functional genomics. Infectious cDNA clones of the genomic RNAs (Viry *et al.*, 1993; Vigne *et al.*, 2013) from two mild GFLV strains (Huss *et al.*, 1989; Legin *et al.*, 1993) were placed under the control of the *Cauliflower mosaic virus* 35S promoter (35S promoter) and delivered to plants using *Agrobacterium tumefaciens* (agroinoculation) to establish infection. The GFLV vector can be used to stably express foreign genetic material, silence endogenous host genes and co-express two heterologous genes. Additionally, the transmission of the GFLV vector by the ectoparasitic nematode *Xiphinema index* was abolished by directed mutagenesis of the coat protein-coding region. The GFLV vector technology furnishes a reliable, versatile and biosafe tool to conduct functional genomics research in grapevine.

## ***MATERIALS AND METHODS***

### **Plant material and inoculations**

*N. benthamiana* was grown in 10cm x 10cm pots in a greenhouse with a 16/8 hr. photoperiod at 24°C ± 3°C. Greenhouse experiments were conducted during fall, lighting was supplemented by high pressure sodium lamps and temperature was automatically controlled by steam-heated radiators and fans, and by ventilator, convection and evaporative cooling systems. Plants were grown in Cornell Mix supplemented with 10-10-10 (N/P/K) fertilizer weekly. GFLV-free *V. vinifera* cv. Cabernet Sauvignon were micropropagated in woody plant medium supplemented with 37 mgL<sup>-1</sup> cysteine contained in polyethylene Star\*Pac<sup>®</sup> bags (Phytotechnology laboratories, Shawnee Mission, KS) with a 16-hr photoperiod at 25 ± 2°C. Grapevines

were agroinoculated at four weeks post-rooting and acclimatized in the greenhouse (Alzubi *et al.*, 2012). GFLV-infected grapevines used for virus quantification were accessed at the USDA Cold Hardy Grapevine Plant Genetics Resource Unit in Geneva, NY. These were the interspecific hybrid Landot noir (one vine) and the rootstock *Vitis rupestris* cv. Alphonse de Serres (two vines). Ten clusters were sampled per vine and tissues from 10 to 20 berries from single clusters were pooled prior to tissue isolation.

All *A. tumefaciens* cultures were grown and inoculated according to the protocol of Vaghchhipawala *et al.* (2010). Individual cultures containing plasmids with GFLV RNA1 or RNA2 cDNA components and silencing suppressors 2b of *Cucumber mosaic virus* (Choi *et al.*, 2008) or p24 of GLRaV-2 (Chiba *et al.*, 2006; Vigne *et al.*, 2013) were mixed and inoculated in equal parts. Leaves of three week-old *N. benthamiana* plants were syringe-infiltrated and grapevine plantlets sonicated for one minute before vacuum infiltration for seven minutes at approximately 90kPa. Mechanical inoculation of GFLV crude sap in *N. benthamiana* and *C. quinoa* was conducted as described (Gottula *et al.*, 2013).

### **Nucleic acid and microbiology procedures**

RNA extraction was accomplished with RN-EZ plant RNA isolation kit from Omega Biotek (Doraville, GA) and reverse transcription and amplification reactions were accomplished with Qiagen One-Step RT-PCR kit according to manufacturer's protocols (Hilden, Germany). Other enzymes and protocols including Phusion DNA polymerase, restriction enzymes and T4 ligase were provided by New England

Biolabs (Ipswich, MA). Primers (**Table 3-1**) were provided by Integrated DNA Technologies (Coralville, IA). GFLV cDNAs from which infectious *in vitro* transcripts were derived (Viry *et al.*, 1993; Vigne *et al.*, 2013) were cloned into CaMV 35S expression cassettes in binary vectors mobilized into *A. tumefaciens* strains GV3101 or C58Z707 via electroporation. RFP was derived from pTagRFP-C (Evrogen) and EGFP was amplified from pEGFP (Clontech). All clones were sequenced (Sanger method) at the Cornell Biotechnology Resource Center (Ithaca, NY) and were comprised of the expected sequence.

### **Cloning strategies**

GFLV-F13 RNA1 cDNA was amplified by PCR from pMV13 (Viry *et al.*, 1993) using primers JG08ForSalRNA1 and JG08RevRNA1Sal and placed in pEPT8 (Vigne *et al.*, 2013) via SalI digestion. The F13 RNA1 cDNA subclone was digested by BglII, fractionated in a 1.5% agarose gel and was ligated into the BglII site of the binary vector pGA482G (designated 'RNA1' in **Figure 3-1B**).

GFLV-F13 RNA1 cDNA was modified for insertion of RFP or EGFP between the 5'UTR and the 1A coding region. This was accomplished by modifying pMV13 clone to contain an AvrII cloning site between the 5' UTR and the 1A coding region by a PCR fusion with primers LR1ST7 and CKAvrNt1AR, and primers CKAvrNt1A and LRRI798, and a SalI and AgeI co-digestion.

Polyprotein P1 was fused to EGFP by PCR amplification with primers CK1ANterAttB1 and CK1ECterAttB2 and successive Gateway recombination in pDonRZeo and pK7GWF2. From this EGFP-1A adjoined construct sequence, EGFP,

**Table 3-1** Primers used in the cloning procedure to engineer GFLV vectors

Table 3-1		
Primer Name	Sequence 5'-3'	
JG08ForSalRNAI	GAGTCGACATGAAAAATTCCACCAAGTTC	
JG08RevRNAISal	GAGTCGACAAAAATTGCATAACAGTAAA	
LR1ST7	TCAGAGTCGACTAATACGACTCACTATAGATGAAAAATTCCACAAGTCTTACG	
CKAvrNtIAR	GGCACCTGCCACATCCTAGGAGTGGCAAACAA	
CKAvrNtI	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGTGGCAGGTGCCTGAGGG	
LRRI798	GGACGACTTTCCTTAGTG	
CKIANterAttB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGTGGCAGGTGCCTGAGGG	
CKIEcterAttB2	GGGGACCACTTTGTACAGAAGAAAGCTGGGTTTATTTCTAAGGCATGTATGATTCTG	
CKAvrNEGFP	GCTCTAGACCTAGGATGGTGAGCAAGGGCGAG	
FBAvrTagRFPF	GGGGTACCCCTAGGATGAGCGAGCTGATTAAAGGAG	
FBAvrTagRFPR	GGGGTACCCCTAGGATTAAGTTTGTGCCCCAGTTTGC	
CKBgI535S	GGAAGATCTGGTACCCCCCTACTCC	
CK335S5'Rlrev	CTTGTGGGAAATTTTCATCCTCTCCAAATGAATG	

### Table 3-1

Primer Name	Sequence 5'-3'
CK335S5'R1sens	CATTTCATTTGGAGAGGATGAAAAATTTCGCCACAAG
CK3'R1BgIrev	GGAAGATCTTTATTAGAAATAAAAAATTTCATACAGTAAAAAAG
JG10SaIGHRNA2For	GGAGTCGACATGAAAAAATTTTGGTAGGTTG
JG10GHRNA2BamHIRev	GGAGGATCCATAAAATTTGC AAAACAG
RLFus1F	GAACTAGGCGCGAATCTTTGATTGG
RLFus1R	CTTGTAGGCACCTGGTTGCACTTAAGTCATATTTCTATGGGAGGCAAGTCAAAAAATTGGAATTTG
RLFus2F	GAAATATGACTTAAGTGCAACACGAGTGCTACAGAAGTGTGGGCTAACTTTGGGCC
RLFus2R	CATACACCCCGGATATTTGAAAAAGTTCAT
RL11MCS2F	AGCACCTTCTGTGTGCTGTGCTACGGACACGCGTACTTATTTCTTGTATTCCTAATTAATCTTCTCTTTATT
AG	
RL11MCS2R	CCACCGGTAGTTGCGCCATCCGCTCCATCTTCAGGAACATAAGGAACTTCTCCTTAATAAAGAAGAAGAA
TAATTAGG	
RL11RNA2F	ATGCTCGAGCGCTTGAATTTCTGATCAAGATCTCCGG

Table 3-1		
Primer Name	Sequence 5'-3'	
RL1IRNA2R	GGGCTCGAGCTGATCAAGATCTTAGTACTGATTTGGTTTAGG	
eGFP <sup>top</sup> ROXF	GAGACGTACGATGGTGAGCAAGGGCGAGGAGC	
eGFP <sup>top</sup> ROXR	GAGAACGCCGTATCCGAGTACTTGTACAGCTCGTCC	
NbPDSBsiWIF	AATCATCGTACGATGCCCCAAATCGGACTTGTATCTGCTG	
NbPDSMluIR	CTCTTAACGCCGTGAATATGTGCAACCCAGTCTCGTACCAATC	
CKSal5'35S	ATCGATGTCGACGGTACCCCCCTACTCC	
CK335S5'R2sens	GTTCAATTTCATTTGGAGAGGATGA AAAAATGTTTACG	
CK335S5'R2rev	CGTAAACATTTTTCATCCTCTCCAAATGA AATGAAC	
CK3'R2PASSalrev	ATCGATGTCGACTTTATTAGAAATAATAAATTTGCAAAACAG	



the attB2 recombination sequence and the 5' part of 1A were amplified with primers CKAvrNtEGFP and LR1ST7 and ligated into pMV13-AvrII following AvrII and AgeI digestion. The TagRFP sequence was introduced into RNA1 by adding an AvrII site at both the 5' and 3' end of the gene using primers FBAvrTagRFPF and FBAvrTagRFPF and PCR amplification and subcloning the AvrII restriction fragment into pMV13-AvrII. The RNA1-EGFP and RNA1-RFP cDNAs were placed upstream of CaMV 35S promoter via overlapping PCRs: the CaMV 35S promoter was amplified from pBin61 with primers CKBgl5'35S and CK3'35S5'R1rev, and RNA1-EGFP was amplified with primers CK3'35S5'R1sens and CK3'R1PASBglrev, fused together and ligated into pUC19 after digestion with BglII-compatible BamHI and the PvuII-PvuII fragment was further subcloned into SmaI-linearized pBin19 to generate 'RNA1-EGFP' and 'RNA1-RFP' (**Figure 3-1B**).

GFLV-GHu RNA2 cDNA was PCR amplified using primers JG10SalIGHRNA2For and JG10GHRNA1BamH1Rev from pG2 and placed in pEPT8 (Vigne *et al.*, 2013) via SalI/BamHI digestion. Site-directed mutagenesis replaced part of the GFLV-GHu RNA2 coat protein (CP) with the nematode non-transmissibility G2 mutant described for GFLV-F13 (Schellenberger *et al.* 2010). The mutation was made via overlapping PCRs with primers RLFus1F and RLFus1R, and primers RLFus2F and RLFus2R, and AvrII and XmaI digestion followed by ligation to replace the intervening site with the synthetic (nematode non-transmissible) CP coding sequence. This GFLV-GHu RNA2 cDNA was further modified to contain unique restriction sites (BsiWI and MluI) between the 2A<sup>HP</sup> and 2B<sup>MP</sup> coding regions, and a synthetic degenerate cleavage site mimicking the R/G site between 1D<sup>Pro</sup>/1E<sup>Pol</sup> was

synthesized upstream of the multiple cloning site (MCS) to allow for maturation of protein 2B<sup>MP</sup>. The MCS and cleavage sequence was synthesized between the 2A<sup>HP</sup>/2B<sup>MP</sup> coding regions using megaprimers RL11MCS2F and RL11MCS2R containing native restriction sites *AleI* and *AgeI* on the 5' and 3' ends, respectively. The CP-modified GFLV-GHu RNA2 clone was digested with *AleI* and *AgeI* and the intervening RNA2 with MCS was inserted. The 35S:RNA2-CP-MCS sequence was then amplified with primers RL11RNA2F and RL11RNA2R, and ligated into binary vector G2b (Choi *et al.*, 2008) via *PspXI* digestion. This clone is designated 'RNA2' in **Figure 3-1B**. The *BsiWI*/*MluI* cloning site on GFLV RNA2 was used to clone EGFP gene after amplification with primers EGFPtopROXF and EGFPtopROXR to generate 'RNA2-EGFP' (**Figure 3-1B**). *N. benthamiana pds* gene fragment was amplified from total RNA of *N. benthamiana* using primers NbPDS*BsiWI*F and NbPDS*MluI*R and ligated into GFLV-RNA2 to generate 'RNA2-pds' (**Figure 3-1B**). The insertion of TagRFP gene into GFLV RNA2 was previously reported (Amari *et al.*, 2010). This clone was placed under the control of a CaMV 35S promoter by overlapping PCRs. The CaMV 35S promoter was amplified from pBin61 with primers CKSa5'35S and CK3'35S5'R2rev, and RNA2-RFP was amplified with primers CK3'35S5'R2sens and CK3'R2PASSalrev, fused together, ligated into *Sall*-digested pUC19, further subcloned into pBin19 via *Sall* digestion and named 'RNA2-RFP'.

### **Protein and fluorescence assays**

GFLV ELISA, immunocapture (IC)-RT-PCR, quantitative fluorescence measurements and t-tests were conducted as described (Gottula *et al.*, 2013; Vigne *et*

*al.*, 2013). Microscopy was accomplished with a Leica SP5 Confocal Microscope (Leica Microsystems, Exton, PA USA) at the Plant Cell Imaging Center at Boyce Thomson Institute for Plant Research at Cornell University (Ithaca, NY). Fresh virus-infected or negative control leaves were fixed in water and illuminated with DIC or Argon lasers. Predefined excitation and emission spectra were used for EGFP and RFP and fluorescence emissions were simultaneously captured with HYD2 detectors using 10X, 20X or 40X objectives. Images were processed using the Leica Application Software Advanced Fluorescence Suite (v 2.6.0) and viewed in ImageJ.

### **Nematode transmission assays**

Wild-type GFLV and GFLV variants based on various RNA1 and RNA2 cDNA constructs were mechanically inoculated to *N. benthamiana*. Infected and control *N. benthamiana* plants were planted into flats containing 300 aviruliferous *X. index* previously reared on fig plants. Nematodes were allowed to feed for six weeks. After acquisition of the virus and removal of the infected *N. benthamiana* plants, nematodes were exposed to healthy *N. benthamiana* or grapevines for eight weeks. Roots of bait *N. benthamiana* and grapevines were tested for GFLV by ELISA and IC-RT-PCR, respectively (Schellenberger *et al.*, 2010).

### **Grapevine agroinfection**

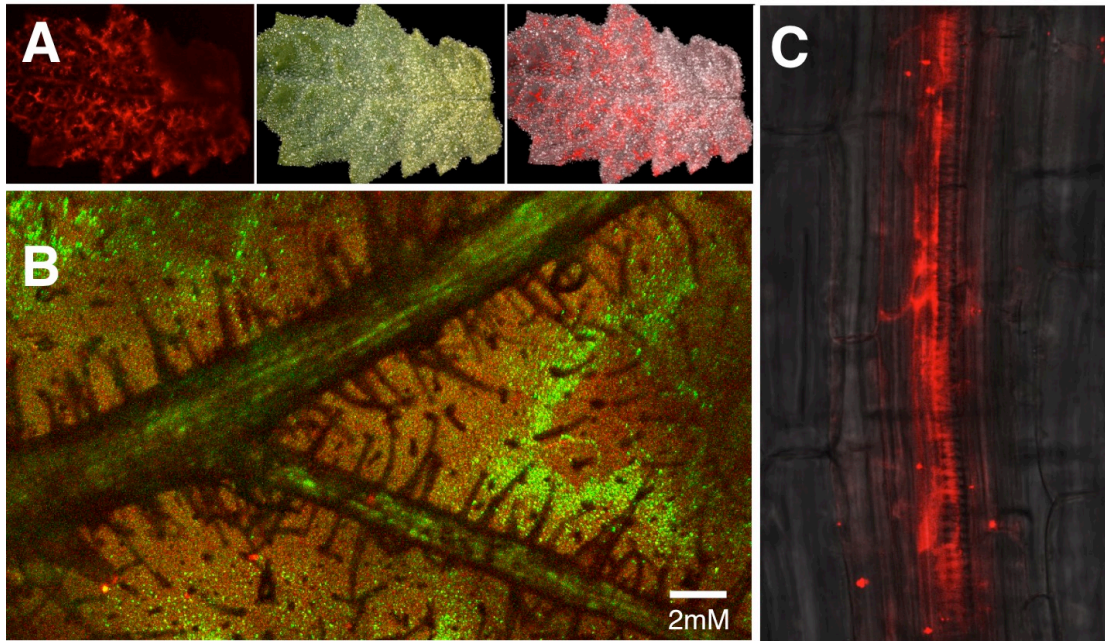
This author undertook seven experiments to test and validate GFLV agroinfection in grapevine. In these experiments, *Vitis* cultivars ('Riesling' F<sub>1</sub> seedlings and 'Cabernet franc' cuttings) were agroinoculated with various GFLV

constructs (**Figure 3-1B** and those in Chapter 4) using syringe and vacuum infiltration. Controls used in these experiments included syringe-infiltrated *Nicotiana benthamiana*, grapevines inoculated with *A. tumefaciens*-free infiltration medium (buffer), and grapevines agroinfiltrated with a GUS-intron construct (Vancanneyt *et al.*, 1990). Young (apical) leaves of grapevines were tested for GFLV by ELISA before and after a dormant period.

## **RESULTS**

### **GFLV can stably express reporter proteins**

Functional cDNA clones of the two genomic RNAs (Viry *et al.*, 1993; Vigne *et al.*, 2013) of GFLV strains F13 and GHu that are attenuated in grapevine (Huss *et al.*, 1989; Legin *et al.*, 1993) were cloned into expression cassettes in binary vectors (**Figure 3-1B**). Homologous or heterologous combination of RNA1 and RNA2 constructs of strains F13 and GHu were agroinoculated to *Nicotiana benthamiana*. Systemic infection in *N. benthamiana* was confirmed for all construct combinations by double antibody sandwich (DAS) enzyme-linked immunosorbent assays (ELISA) two weeks post-agroinoculation (data not shown). Reporters *TagRFP*, a derivative of *Red fluorescent protein (RFP)* (Merzlyak *et al.*, 2007) and *enhanced Green fluorescent protein (EGFP)* (Clontech) were tagged between the 2A<sup>HP</sup> and 2B<sup>MP</sup> coding regions of GFLV RNA2 (**Figure 3-1B**) and co-agroinoculated with GFLV RNA1. The same reporter genes were also cloned upstream of the 1A coding region of GFLV RNA1 (**Figure 3-1B**) and co-infiltrated with GFLV RNA2. Agroinoculated *N. benthamiana* plants became systemically infected, as indicated by DAS-ELISA, and expression of



**Figure 3-2** Fluorescence expression in plants infected with the *Grapevine fanleaf virus* (GFLV) vector. A) Composite image of a *Chenopodium quinoa* leaf showing expression and distribution of RFP expressed from GFLV RNA2 at nine days post-inoculation (dpi). From left to right: images of RFP, transmitted light, and overlaid both. B) The GFLV vector expressing enhanced Green fluorescent protein (EGFP) in a young apical *Nicotiana benthamiana* leaf at 15 dpi. Red represents chlorophyll autofluorescence and green represents EGFP. C) RFP expression from a GFLV vector encoding RFP in a *N. benthamiana* root cortex.

**Table 3-2** Stability of *Red fluorescent protein* (RFP) expression from the GFLV vector in *Nicotiana benthamiana* and *Chenopodium quinoa*.

	<i>Nicotiana benthamiana</i>		<i>Chenopodium quinoa</i>	
Infection	Infection rate <sup>a</sup>	RFP expression <sup>b</sup>	Infection rate <sup>a</sup>	RFP expression <sup>b</sup>
1st passage	2/5	2/5	6/13	6/13
2nd passage	2/2	2/2	8/8	8/8
3rd passage	2/2	2/2	15/15	15/15
4th passage	2/2	2/2	15/15	15/15

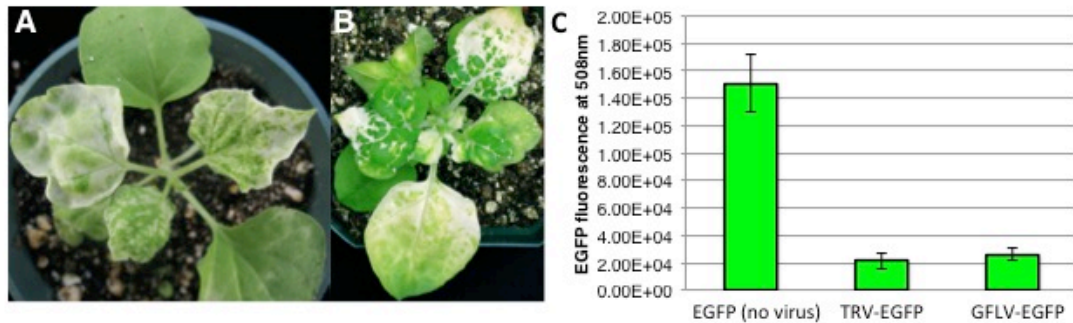
<sup>a</sup> Total number of ELISA positive plants for GFLV over the total number of inoculated plants. After initial infection in *N. benthamiana* and each passage, one RFP (fluorescence microscopy) and GFLV positive *N. benthamiana* (ELISA) was used as inoculum at two weeks post-inoculation for each subsequent passage. N/A: not applicable.

<sup>b</sup> Total number plants expressing red fluorescence assessed by confocal microscopy and a microplate read over the total number of plants tested.

EGFP was observed in leaves (**Figure 3-2B**). Expression of EGFP was also observed in leaves and roots of *Chenopodium quinoa* following mechanical inoculation that used sap of agroinfected *N. benthamiana* (**Figure 3-2A and 4-2C**). The stability of the RFP-tagged GFLV vector was determined by four serial passages in *N. benthamiana* and *Chenopodium quinoa* at two-week intervals following agroinfection of *N. benthamiana* (**Table 3-2**). The presence of RFP in every infected plant following four serial passages is promising, given that other viral vectors undergo insert deletions in zero to four passages (Fernández-Fernández *et al.*, 2001; Haviv *et al.*, 2006; Touriño *et al.*, 2008). These results indicated GFLV vectors can stably express foreign genetic material in plant leaves and roots.

### **GFLV is an effective vector for silencing endogenous genes**

Because null mutants are not always available for gene complementation assays, knockdown of host genes of known sequence is key for functional genomics research. Therefore, we tested the GFLV vector for capability of virus-induced gene silencing (VIGS) and compared it to the widely used VIGS vector *Tobacco rattle virus* (TRV) (Ratcliff *et al.*, 2001). To this aim, we cloned a partial fragment (501bp) of *phytoene desaturase* (*pds*) sequence from *N. benthamiana* between the 2A<sup>HP</sup> and 2B<sup>MP</sup> coding regions of GFLV RNA2 (**Figure 3-1B**) and agroinoculated *N. benthamiana* with GFLV RNA1 in a comparative test to the TRV vector with a 402bp *pds* insert. At nine days post-inoculation (dpi), the expected photobleaching phenotype was obtained (**Figure 3-3A and 3-3B**) and maintained for up to 120dpi. In order to quantify VIGS efficacy, we inoculated *N. benthamiana* constitutively expressing high levels of EGFP

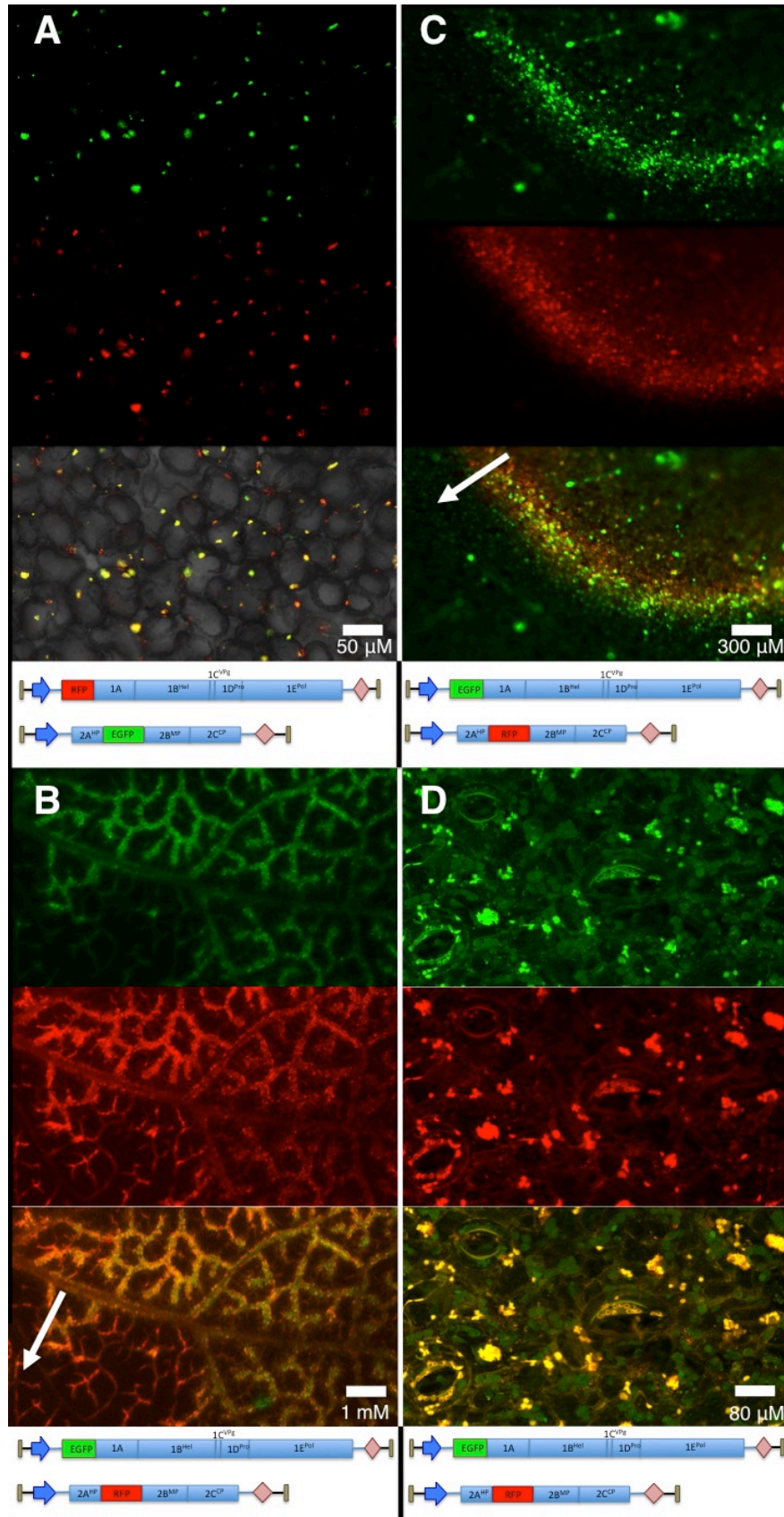


**Figure 3-3** Comparative virus-induced gene silencing activity of the *Grapevine fanleaf virus* (GFLV) and *Tobacco rattle virus* (TRV) vectors for silencing *phytoene desaturase* (pds) and *enhanced Green fluorescent protein* (EGFP) expression in *Nicotiana benthamiana*. A) GFLV-pds-induced photobleaching versus B) TRV-pds-induced photobleaching phenotype at 14 days post-inoculation (dpi). C) Quantitative comparison of GFLV and TRV-induced silencing of EGFP in EGFP transgenic plants at 14dpi. Error bars represent standard error of the mean (n=3).





**Figure 3-4** Dual gene expression patterns of the *Grapevine fanleaf virus* vector. Red fluorescent protein (RFP, red) and enhanced Green fluorescent protein (EGFP, green) genes were inserted in either GLFV RNA1 or RNA2. Each subject is represented for EGFP fluorescence (top segments), RFP fluorescence (middle segments) and the EGFP-RFP overlay (lower segments). GFLV vectors are indicated at the bottom of each panel and arrows show the direction of the infection front in panels B and C. A) Infection sites are present at three days post-inoculation (dpi) in agroinoculated *Nicotiana benthamiana* cells expressing RNA1-RFP and RNA2-EGFP. B) Patterns of FP expression in upper non-inoculated leaves for RNA1-RFP and RNA2-EGFP in *C. quinoa*. C) Patterns of FP expression at the front of infection for RNA1-EGFP and RNA2-RFP in *N. benthamiana* at 6 dpi. D) Co-expression of RNA1-EGFP and RNA2-RFP showing 1A-2A<sup>HP</sup> protein aggregates in a grapevine leaf at nine dpi.

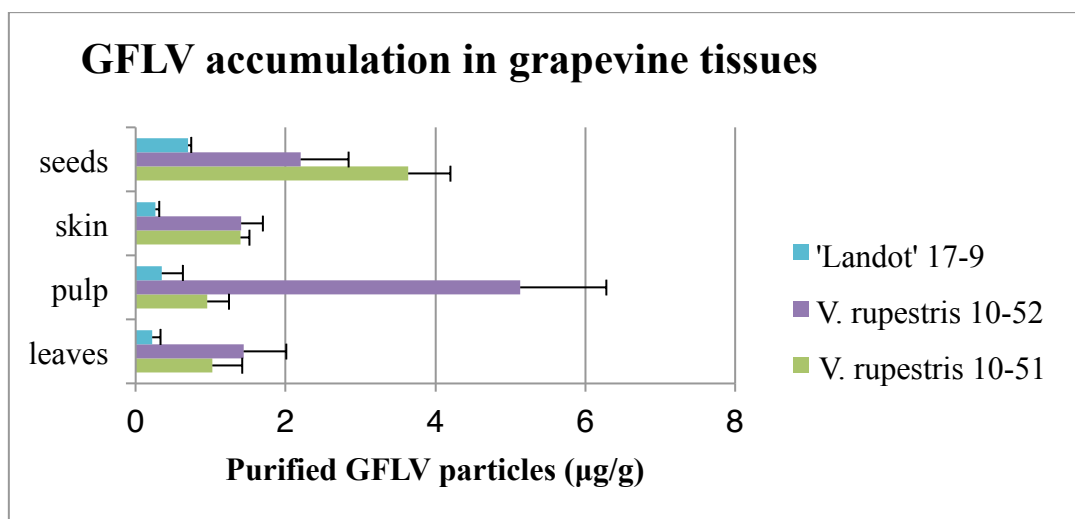


fluorescence at the front of infection in *N. benthamiana* epidermal cells (**Figure 3-4C**). These observations indicated that RNA1 is able to express its heterologous insert in advance of RNA2, which reflects the dependence of RNA2 polyprotein maturation on the RNA1-encoded proteinase (Margis *et al.*, 1994). These experiments showed GFLV vector can be used to concurrently express two heterologous genes and can be used for tandem or sequential gene expression experiments when multiple genes are to be functionally characterized.

The EGFP and RFP-tagged GFLV vector was agroinoculated to ten plants of *Vitis vinifera* cv. Cabernet Sauvignon. Agroinoculated leaves revealed expression of EGFP and RFP by nine dpi in eight of ten plants. Confocal microscopy images indicated EGFP and RFP co-expression in multiple cells in aggregates characteristic of 1A-2A<sup>HP</sup> co-localization (**Figure 3-4A and 3-4D**). Guard cells, epidermal cells and mesophyll tissue contained EGFP and RFP overlaid, and a few guard cells contained solely 1A-TagRFP aggregates. Expression of the EGFP and RFP-tagged GFLV aggregates were confirmed through 30dpi in inoculated leaves.

### **GFLV expresses proteins at high levels in grape berries**

Because grapevines generally require three years to fruit, we tested naturally GFLV-infected grapevines for virus titer in berry tissues. Grape clusters from three infected grapevines were harvested at maturity and the seeds, skin and flesh was manually separated and tested by semi-quantitative DAS-ELISA. Results indicate that GFLV accumulates in the different berry tissues tested (**Figure 3-5**). A comparative analysis of relative GFLV titer shows an approximately 100-fold lower accumulation



**Figure 3-5** Dosage of *Grapevine fanleaf virus* (GFLV) in leaves and berries of naturally infected grapevines. Tissue from the interspecific Landot and *V. rupestris* was tested for GFLV by semi-quantitative DAS-ELISA. Error bars represent standard error of the mean (n=4).

in grapevine berry and leaf tissue (**Figure 3-5**) than in systemic herbaceous hosts (Vigne *et al.*, 2013), but a similar accumulation compared to other viral vectors in herbaceous hosts (Fernández-Fernández *et al.*, 2001; Li *et al.*, 2004; Yoon *et al.*, 2011; Chen *et al.*, 2012). This indicates the GFLV vector will be suitable for functional genetics testing of grapevine berries and can be used to test genetic hypotheses in each of the two genomic layers of grapevines (Reisch *et al.*, 2011).

### **The GFLV vector is abolished for nematode vector transmission**

A growing interest in using viral vectors as tools for vaccination of perennial crops (Dawson and Folimonova, 2013) necessitates the addition of a biosafety component. Since GFLV is vectored specifically by the ectoparasitic nematode *Xiphinema index* and the viral determinant of nematode transmission is well characterized (Schellenberger *et al.*, 2010; 2011), we conducted site-directed mutagenesis of 11 amino acids that are responsible for transmission and are located within the GFLV vector-RNA2-encoded coat protein coding region (Protocol S1). The vector and controls, including transmissible and non-transmissible GFLV strains (Schellenberger *et al.*, 2010), were deployed in *X. index* transmission assays following agroinoculation of *N. benthamiana*. Transmission assays showed that the GFLV vector is *X. index* non-transmissible (**Table 3-3**). This indicates the GFLV vector could be deployed in vineyards or other low-containment settings without concern that it will become naturalized through *X. index*-mediated transmission, unless recombination with the RNA genome of wild-type strains would restore transmissibility.

### **Lack of grapevine systemic infection following agroinfiltration**

Grapevine plants were agroinfiltrated with GFLV or Gus-intron controls. Excellent Gus-intron expression was verified at nine days post-inoculation in multiple experiments (data not shown). ELISA tests for GFLV before and after dormant periods revealed no instance of systemic GFLV infection following agroinfiltration in seven experiments where 323 plants were used. Local GFLV infection was observed using fluorophor-tagged RNA1 and RNA2 constructs derived from GFLV-F13 (**Figure 3-4D**), but likewise these plants did not become systemically infected according to ELISA and fluorescence microscopy.

### ***DISCUSSION***

Functional genomics platforms based on viral vectors have numerous advantages compared to stable transformation or transient expression systems including reduced time from concept to phenotype, greater ease of use, grander scalability potential and often greater consistency within treatments. However, viral vectors have weaknesses compared to other functional genomics systems including a frequent lack of stability, tissue specificity, relatively low carrying capacity of genetic information, host-pathogenic effects and concerns about environmental release of the infectious agent. Therefore, although dozens of viral vectors have been validated, few have been widely used in economically important crops, and even fewer in perennial woody crops (Dawson and Folimonova, 2013). A few research groups have developed viral vectors for grapevine, but each of these vectors comes with some limitations including tissue specificity (GLRaV-2, GVA and GRSPaV are phloem limited) and

**Table 3-3** Transmissibility of the *Grapevine fanleaf virus* (GFLV) vector by *Xiphinema index*.

GFLV treatment <sup>a</sup>	<i>Nicotiana benthamiana</i> <sup>b</sup>	<i>Vitis</i> <sup>b</sup>
Agroinoculated GFLV (wt)	19/48	25/48
Agroinoculated GFLV (vector)	0/45	0/47
Transmissible GFLV-F13	19/24	13/22
Nontransmissible GFLV-AG2	0/19	0/17
Mock	0/9	0/9

<sup>a</sup> GFLV strains were derived from agroinoculation using wild-type (wt) or vector cDNA carrying the CP mutation, or previously described transmissible GFLV-F13 or non-transmissible GFLV-AG2 (Schellenberger *et al.*, 2010).

<sup>b</sup> Data represent the number of bait plants positive for GFLV in ELISA (*N. benthamiana*) or IC-RT-PCR (*Vitis*) over the number of plants used as bait.



possibility of unintended release [very little information is known about GVA transmission by mealybugs and no vector is known for GLRaV-2 and GRSPaV]. Additionally, GFLV is based on a bipartite RNA genome, which can facilitate spatially phased expression of heterologous proteins, unlike vectors based on viruses with monopartite RNA genomes such as GLRaV-2, GVA and GRSPaV.

The GFLV vectors capture all of the advantages and limits most or all disadvantages of other grapevine viral vector technologies in the following ways: the vector (1) is easy to use given that GFLV-RNA2 can be modified to contain inserts based on restriction enzyme digestion (BsiWI and MluI) in the small modular binary vector pGreen, (2) can stably carry inserts, (3) can efficiently silence endogenous genes, (4) can concurrently or simultaneously express two foreign sequences depending on the spatio-temporal position of virus infection and (5) cannot be transmitted by its ectoparasitic nematode vector. In addition, GFLV is expressed to high levels in all tissues of grapevine (Andret-Link *et al.*, 2004), including all parts of berries (**Figure 3-5**). For these reasons, the GFLV vector could be an ideal functional genomics platform for grapevine.

The present generations of the GFLV vector have the critical limitation of not producing systemic infection in grapevine following GFLV cDNA agroinfiltration. The lack of grapevine systemic infection could relate to one of several issues with the constructs. The first hypothesis is that the GFLV transcripts derived from cDNAs used are not infectious in grapevine, as was the case with precursory versions of the *Grapevine leafroll associated virus-2* vector (Liu *et al.*, 2009; Kurth *et al.*, 2013). This hypothesis is unlikely because the cDNAs derived from GFLV-F13 and GFLV-GHu

(i.e. FF and GG, Chapter 4) are infectious in grapevine following heterologous grafting (E. Vigne, personal communication). Another possible explanation of the lack of systemic infectivity in grapevine could relate to the tagging of many GFLV transcript constructs (e.g. those used in Chapter 4) with the *Alfalfa mosaic virus* RNA4 translational enhancer at their 5' ends. Because fidelity of 5' ends of RNA viruses including GFLV is critical to their infectivity (Chapter 2; Liu *et al.*, 1991; Annamalai *et al.*, 2003), it is plausible that this structure comprises an impediment to infectivity of GFLV transcripts following agroinfiltration.

Provided that future generations of the GFLV vector could be successfully inoculated to grapevine, this vector could be utilized in ways similar to vectors validated for several other crop species. In wheat, *Barley stripe mosaic virus* (BSMV)-based vectors were used to knock down expression of candidate genes for powdery mildew resistance and these functional analyses conclusively identified *Triticum aestivum* powdery mildew resistance 5 and mildew resistance locus O as bases of resistance (Várallyay *et al.*, 2012; Yuan *et al.*, 2012). It can be envisioned that the GFLV vector could be used to individually down regulate expression of eight candidate genes contained in the *resistance to Uncinula necator* 1 linkage group associated with powdery mildew resistance (Dry *et al.*, 2010). In another study, a BSMV vector was used to identify (or disprove) involvement of several candidate genes in drought resistance in wheat (Manmathan *et al.*, 2013). Similarly, the GFLV vector could be deployed to better understand the role of aldehyde dehydrogenases for drought tolerance in grapevine (Zhang *et al.*, 2012). The stability, ability to express novel or silence endogenous genes, and lack of tissue tropism in the GFLV vector

makes it ideal to study genes responsible for biotic and abiotic stress tolerance in grapevines.

Deployment of the GFLV vector could facilitate the selection of grapevines with optimal flavor profiles among breeding populations. Numerous genes have been identified as candidates for grape flavor (Battilana *et al.*, 2011; Dunlevy *et al.*, 2013) however, conclusive proof by an effective complementation system remains elusive and functional analysis in grapevine is necessary before surefire marker-assisted selection tools can be developed. Some viral vectors have extraordinary versatility in that they support the expression of two genes (Roy *et al.*, 2011; Kurth *et al.*, 2012) and the two gene expression capability of the GFLV vector should be particularly handy because it could be used to parse out interconnected and occasionally redundant biochemical pathways that create grape flavor profiles (Dai *et al.*, 2011). The facts that GFLV can express high levels of protein in grape skin and pulp and express proteins in a phased manner illustrate its potential utility for modifying these pathways.

Grape growers are increasingly dismayed by the growing prevalence of insect-vectored bacterial diseases such as Pierce's Disease for which no effective control measures exist. Plant pathologists and entomologists have responded by positing the use of viral vectors as tools for vaccination against the agents of disease or the insects that carry them (Folimonova and Dawson, 2013; Karthikeyan *et al.*, 2013; Gu and Knipple, 2013). Recent proofs-of-concept have shown that viral vectors can effectively silence insect genes and kill insects (Khan *et al.*, 2013; Wuriyanghan and Falk, 2013). Foundational work on plant-expressed diffusible signal factor and antibacterial protein chimeras has demonstrated the feasibility of attenuating *Xylella*

*fastidiosa* infection in grapevines (Chatterjee *et al.*, 2008; Dandekar *et al.*, 2012). The GFLV vector could theoretically be used to deliver proteins with insecticidal, antibacterial or even antiviral activities to grapevines in vineyard situations, with assurances that pathogenic effects of the vector is minimal (given the attenuation of the strains used here) and that the vector would not be disseminated to neighboring grapevines by *X. index*-mediated transmission.

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## CHAPTER 4

### ***GENOMIC, ENVIRONMENTAL AND HOST VARIABLES INFLUENCING GRAPEVINE FANLEAF VIRUS AGROINFECTION***

#### ***ABSTRACT***

Parameters that support local and systemic infection of *Grapevine fanleaf virus* (GFLV), a bipartite RNA virus from the genus *Nepovirus*, family *Secoviridae*, in *Nicotiana benthamiana* following *Agrobacterium tumefaciens*-mediated delivery were tested. The cDNAs of GFLV strains F13 and GHu and recombinants derived thereof were reassorted and tested alongside cDNAs of wild type strains for infectivity. Measures of transient GFLV accumulation following agroinfiltration did not always reflect differential systemic infectivity of the GFLV genomes, but a synthetic assortment of F13 RNA1 and GHu RNA2 cDNAs consistently showed the highest rate of local and systemic infection. Multiple experiments that iteratively tested individual variables showed that systemic GFLV infection was correlated with lower ambient greenhouse temperatures, reduced *A. tumefaciens* suspension density and co-infiltration with silencing suppressors, but not with *A. tumefaciens* strain identity, co-culture with acetosyringone or plant size. Agroinfiltrating transgenic plants expressing either RNA1 or RNA2 cDNAs with a complementary genome part established systemic infection but at a lower rate relative to transiently expressed counterparts. Finally, agroinoculating a panel of *Nicotiana* species showed differential GFLV accumulation in patch assays that could not be explained by *A. tumefaciens* transient expression capabilities or status of resistance to mechanical inoculation, suggesting



complex virus-host interactions. This research shed light on factors governing the success of *A. tumefaciens*-mediated delivery of GFLV for *Nicotiana* infection.

## **INTRODUCTION**

Since Grimsley *et al.* (1987) reported the first use of *Agrobacterium tumefaciens* as a tool to deliver viruses to plants, there has been an explosion of publications on the creation and development of viral vectors (Senthil-Kumar and Mysore, 2011; Dawson and Folimonova, 2013). *A. tumefaciens*-mediated virus inoculation (agroinfection) has been developed for various crops including orchids (Hsieh *et al.*, 2013), monocot field crops (Yuan *et al.*, 2011), tree fruit crops (Ambros *et al.*, 2011) and vegetable crops (Delbianco *et al.*, 2013). Viral vectors have been used for very diverse research endeavors including expressing metabolites (Zhang *et al.*, 2013) or MYB transcription factors (Bedoya *et al.*, 2012), and silencing bacterial resistance and susceptibility genes (Balaji *et al.*, 2011). Viral vectors have been put to use in allelism tests (Fitzgerald *et al.*, 2012), to produce heterologous antigens for vaccines (Gleba *et al.*, 2007; Thuenemann *et al.*, 2013), to deliver insect-disruption molecules (Wuriyanghan and Falk, 2013) and to facilitate stable genome modification (Kopertekh *et al.*, 2012). In 28 years, viral vectors have moved from proof-of-concept technologies to widely used research tools and may have a future in direct agronomic applications (Dawson and Folimonova, 2013).

Compared to substitute plant biotechnologies, viral vectors have both advantages and limitations. Viral vectors can produce large amounts of heterologous protein or efficiently knock down mRNA expression 90% or more (Kagale, 2012; Ma *et al.*, 2012). Viral vectors can reveal functional phenotypes faster than stably transformed plant lines (Pogue *et al.*, 2002). On the other hand, viral vectors exhibit limitations on the amount of genetic material they can stably replicate (Senthil-Kumar

and Mysore, 2011), they can be destructive in controlled greenhouse situations if the live agent is unintentionally disseminated (Hayward *et al.*, 2011), and may not be fully reliable due to lack of infectivity (Liu *et al.*, 2009). Improving viral vector infectivity is often quite challenging because only a handful of mutations can render a virus noninfective in a given host (Kurth *et al.*, 2012).

*Grapevine fanleaf virus* (GFLV) from the genus *Nepovirus*, family *Secoviridae* has a bipartite genome composed of single stranded positive-sense RNA molecules (Sanfaçon *et al.*, 2009). Each genome segment contains a viral genome-linked protein (VPg) at the 5' end and a poly-A tail at the 3' terminus, and is translated into a polyprotein. RNA1 (7.3kb) contains the materials necessary and sufficient for replication and protein maturation, including a helicase, proteinase, VPg, RNA-dependent RNA polymerase, and a protein (1A) of an unknown function. RNA2 (3.7kb) encodes the movement protein and coat protein, and the 2A<sup>HP</sup> protein responsible for RNA2 replication in conjunction with RNA1 replicase proteins (Andret-Link *et al.*, 2004). Both GFLV genomic RNAs are required for systemic plant infection (Andret-Link *et al.*, 2004). The virus is specifically vectored by the dagger nematode *Xiphinema index*, and is host-specialized to grapevine and a few herbaceous hosts including *Nicotiana benthamiana*. Mechanically inoculation with infectious sap or transcripts derived from full-length cDNA clones leads to GFLV infection in *N. benthamiana* (Valat *et al.*, 2003). Recently, infectious GFLV cDNA clones corresponding to the genomic RNAs of strain F13 (Viry *et al.*, 1993) and GHu (Vigne *et al.*, 2013) were placed downstream of the *Cauliflower mosaic virus* (CaMV) 35S promoter into binary vectors suitable for *A. tumefaciens*-mediated infection *in planta*

(Gottula *et al.*, 2014a). The GFLV vector can simultaneously express two heterologous proteins, efficiently silence plant genes through VIGS, and is unable to be transmitted by *X. index* as a result of a targeted mutation in the coat protein (Gottula *et al.*, 2014a). In the present manuscript, variables tested to optimize conditions for agroinfection of GFLV in *N. benthamiana* are reported.

## **MATERIALS AND METHODS**

### **Molecular cloning procedures**

All enzymes, buffers and protocols were from New England Biolabs (NEB, Ipswich, MA). This included PCR materials (Phusion DNA Polymerase), reverse transcriptase (AMV), T4 DNA ligase, and several restriction enzymes. All plasmids were transformed into chemically competent *Escherichia coli* strain DH5 $\alpha$ , which were grown in agar-containing or liquid Luria-Bertani (LB) medium at 37°C. The pEPT8-derived plasmids (Ling *et al.*, 1997; Gottula *et al.*, 2014a) were selected with ampicillin (100 $\mu$ g/mL), pGreenII- (Choi *et al.*, 2008) and pROK2- (Gottula *et al.*, 2014a) based plasmids with kanamycin (50 $\mu$ g/mL), and pGA482G-based plasmids (Chee *et al.*, 1989) with gentamicin (100 $\mu$ g/mL). Primers were from Integrated DNA Technologies (Iowa City, IA) (**Table 4-1**). Plasmids were extracted with Omega Miniprep Kit (Norcross, GA) and Sanger sequencing was accomplished at Cornell Biotechnology Resource Center (Ithaca, NY).

### **GFLV vector and silencing suppressors**

Previously validated cDNAs of two GFLV strains were cloned downstream of

the CaMV 35S promoter in binary vectors. The cDNAs of RNA1 and RNA2 of strains GFLV-F13 (Viry *et al.*, 1993) and GFLV-GHu (Vigne *et al.*, 2013) were subcloned into pEPT8 and into the binary vector pGA482G (Chee *et al.*, 1989) as described by Gottula *et al.* (2014a) and in Supplementary Protocol 1. These clones were used to generate four GFLV genomes, i.e. FF, GG, FG and GF, named on the basis of the F13- or GHu- composition of the RNA1 and RNA2 constructs (**Table 4-2**).

Additionally, two GFLV RNA2 recombinants were generated: The F<sub>G2A</sub> construct had the 2A<sup>HP</sup> protein coding sequence of GFLV-GHu placed in the GFLV-F13 RNA2 background; the other RNA2 construct G<sub>R</sub> is comprised of GFLV-GHu RNA2 with a multiple cloning site composed of BsiWI and MluI restriction sites, and a site-directed coat protein mutation for abolishment of *X. index* transmissibility (Gottula *et al.*, 2014a) (**Table 4-2**). All GFLV constructs were present in the CaMV 35S expression cassette (35S promoter and terminator) from pEPT8. All GFLV constructs were engineered into the CaMV 35S expression cassette (35S promoter and terminator) from pEPT8. All GFLV constructs were contained in the binary vector pGA482G, except the G<sub>R</sub> construct was contained in the modular binary pGreenII-0229 that also contains a *Cucumber mosaic virus* (CMV) 2b expression cassette (Choi *et al.*, 2008). The different GFLV constructs in *A. tumefaciens* vectors, i.e. FF, GG, GF, FG, FF<sub>G2A</sub>, GF<sub>G2A</sub>, FG<sub>R</sub>, GG<sub>R</sub>, are referred to herein as ‘genomes’ for simplicity. The cloning of F13 RNA1 cDNA and G<sub>R</sub> was previously described, where Chapter 3 denoted F13 RNA1 cDNA as ‘RNA1 cDNA’ and G<sub>R</sub> was previously denoted ‘pROX’.

Silencing suppressor constructs CMV 2b (Choi *et al.*, 2008) and *Tomato bushy stunt virus* (TBSV) p19 (Canto *et al.*, 2006) were used to test whether heterologous

**Table 4-1** Primers used for the modification of Grapevine fanleaf virus cDNAs and their placement in binary vectors

Primer #	Primer name	Sequence 5'-3'
1.	EVG32	AGAGCTGCGGCACTCTTTC
2.	EVG33	AGMAGAWCCAATAAAGGCGG
3.	JGnpII5F	GGAATACTGCCTAGCACCGT
4.	JGnpII5R	GACATCCTCCAACGAGCATA
5.	JG08ForSalRNA2	GAGTCGACATAAAATTTGC AAAACAGTAAAAAGAAAGG
6.	JG08RevRNA2Sal	GAGTCGACGATGAAAAAATTTTACGTTTCTTACG
7.	JG10SalIGHRNA1IFor	GGAGTCGACATGAAAAAATTTCTACAGGTTTC
8.	JG10GHRNA1INotRev	GGAGCGGCGGCTAAATTTGC AAAAACAGT
9.	JG10SalIGHRNA2For	GGAGTCGACATGAAAAAATTTTGGTAGGTTTC
10.	JG10GHRNA2BamHIRev	GGAGGATCCATAAAATTTGC AAAAACAG
11.	JG10ForBglSaxXbapEPBB2	GGAAGATCTGAGCTCTCTAGATAGTACTGATTTGGTTTAGG
12.	JG10RevpeEPBBXbaSacBgl	GGAAGATCTGAGCTCTCTAGACCGGGCAATTGAGACTTTTC
13.	JG08ForEcoBclBglpEPT8	GAGAAATCTGATCAAGATCTTAGTACTGATTTGGTTTAGG
14.	JG08RevpePT8BglBclEco	GAGAAATCTGATCAAGATCT
15.	JG10SalIRFPFor	GGAGTCGACATGAGCGAGCTGATTAAAGGAGAACATGC
16.	JG10RFPNotRev	GGAAGCGCGGCTCACTTGTGCCCCAGTTTGCTAGGG

**Table 4-2** Nomenclature of the GFLV genomes used in Chapter 4

Genome name	GFLV strain of	
	RNA1	RNA2 <sup>a</sup>
FF	F13	F13
FG	F13	GHu
GF	GHu	F13
GG	GHu	GHu
FF <sub>G2A</sub>	F13	F13(GHu 2A)
GF <sub>G2A</sub>	GHu	F13(GHu 2A)
FG <sub>R</sub>	F13	recombinant GHu
GG <sub>R</sub>	GHu	recombinant GHu

<sup>a</sup> F13(GHu 2A) denotes that the 2A coding sequence of GFLV-F13 was replaced by that of GFLV-GHu. Recombinant GHu denotes modifications including insertion of a multiple cloning site between the 2A and 2B cDNAs, and a site-directed mutation to the coat protein-coding sequence.

silencing suppressors affect GFLV protein accumulation and systemic infection. CMV 2b is contained in the pGreenII-0229 binary vector and TBSV p19 is in the pROK2 binary vector. The negative controls for silencing suppressor assays were fluorescent proteins enhanced Green fluorescent protein (eGFP) and Red fluorescent protein (RFP) expressed from CaMV 35S cassettes in pGA482G. The construction of the eGFP clone was previously reported (Vigne *et al.*, 2013).

### **Specific cloning strategies for the GFLV vectors used in this study**

GFLV cDNAs in T7 expression cassettes that were previously verified to be functional for plant inoculation (pVECP2, pG1 and pG2) (Viry *et al.*, 1993; Vigne *et al.*, 2013) were amplified by PCR and ligated into cassettes containing a *Cauliflower mosaic virus* (CaMV) 35S expression cassette containing a duplicated enhancer (pEPT8) (Ling *et al.*, 1997) or modified versions of pEPT8 (Vigne *et al.*, 2013). The cloning of F13 RNA1 cDNA and mutated GHu RNA2 cDNA (G<sub>R</sub>, alias pROX) was reported in Chapter 3. The GFLV-F13 RNA2 cDNA was amplified by PCR using primers P5 and P6 (**Table 4-1**) from pVECP2 and cloned into pEPT8<sub>BB</sub> with Sall to generate RNA2-17. The F13 RNA2 with the GHu 2A<sup>HP</sup> replacement was created from plasmid pGORF2 digested with AgeI and AlwNI followed by ligation of the corresponding fragment into plasmid pF2 (Vigne *et al.*, 2013). The RNA2 cDNA was amplified from the modified pF2 with primers P5 and P6 and cloned into pEPT8<sub>BB</sub> as described for RNA2-17 to generate 2ABB. The GHu RNA1 cDNA was cloned into pEPT8<sub>MCS2</sub> with Sall and NotI after amplification with primers P7 and P8 to generate 1M2. The GHu RNA2 cDNA was cloned into pEPT8<sub>MCS2</sub> with Sall and BamHI after



amplification with primers P9 and P10 to generate 2MCS20.

The binary vector used to propagate and deliver GFLV cDNAs is a derivative pGA482 modified to contain a gentamycin selectable marker, designated pGA482G (Ling *et al.*, 1997). The T-DNA of pGA482G contains unique restriction sites BglII and XbaI, which were used to insert individual GFLV cDNAs in the CaMV 35S cassettes. The RNA1 cDNA of GFLV strain GHu in the CaMV 35S cassette (1M2) was amplified with primers P11 and P12 and ligated into pGA482G using XbaI to generate GHu RNA1 cDNA. RNA2 cDNAs of GFLV F13-based clones (RNA2-17 and 2ABB) and GHu RNA2 (2MCS20) and their expression cassettes were amplified via PCR with primers P13 and P14, digested with BclI, and cloned into the overhang compatible BglII site in pGA482G to generate F13 RNA2 cDNA, F<sub>G2A</sub> and GHu RNA2 cDNA.

RFP (Merzlyak *et al.*, 2007) was amplified with primers P15 and P16 and inserted into pEPT8<sub>MCS2</sub> after restriction digestion of insert and vector with SalI and NotI. The RFP and 35S expression cassette sequence was amplified with primers P9 and P10, digested with BglII and cloned into the BglII site of pGA482G. Confocal microscopy revealed this clone produced fluorescence following agroinfiltration (data not shown).

Integrity of selected recombinant clones was verified by sequencing, and in all cases no modification of the GFLV cDNA was observed. Mutations in the TATA box were observed in the promoters of each cassette with pEPT8 (TATATAA to TATATAT), but high levels of expression have been verified from clones resulting from this construct (Vigne *et al.*, 2013). An unanticipated consequence of PCR

amplification of the expression cassettes was the truncation of the duplicated CaMV 35S enhancer motifs to a single promoter in GHu RNA1, GHu RNA2, and F<sub>G2A</sub> clones.

Silencing suppressor constructs CMV 2b (Choi *et al.*, 2008) and *Tomato bushy stunt virus* (TBSV) p19 (Canto *et al.*, 2006) were used to test whether heterologous silencing suppressors affect GFLV protein accumulation and systemic infection. CMV 2b is contained in the pGreenII-0229 binary vector and TBSV p19 is in the pROK2 binary vector. The negative controls for silencing suppressor assays were fluorescent protein sequences *enhanced Green fluorescent protein (eGFP)* and *Red fluorescent protein (RFP)* cloned in pGA482G in CaMV 35S expression cassettes. The construction of the eGFP clone was previously reported (Vigne *et al.*, 2013). RFP (Merzlyak *et al.*, 2007) was subcloned into pEPT8 and the resulting 35S:RFP cloned into pGA482G.

### ***Agrobacterium tumefaciens* strains and agroinfiltration**

Electrocompetent cells of *A. tumefaciens* strains C58Z707 (C58), LBA4404 + pSB1 (LBA4404) and GV3101 + pMP90 (GV3101) were transformed via electroporation with individual binary vectors and all transformants were selected with gentamicin (100µg/mL) plus kanamycin (50µg/mL). Strains C58 and LBA4404 were used for pGA482G-based clones, and GV3101 was used for pGreenII- or pROK2-based clones. The helper binary pClean-S161 was used in conjunction with the pGreenII plasmids (G<sub>R</sub> and CMV 2b). The identity of the binary vectors in each *A. tumefaciens* stock was confirmed by plasmid extraction and restriction digestion.

Agroinfiltration was done according to Vaghchhipawala *et al.* (2010) except that all *A. tumefaciens* cultures were prepared to an initial density of OD<sub>600nm</sub>=1.0. GFLV (RNA1 and RNA2) and heterologous silencing suppressor (p19 and 2b) cultures were mixed together in equal ratios unless otherwise noted. *N. benthamiana* plants were infiltrated with a needleless syringe in two leaves (Vaghchhipawala *et al.*, 2010). Several systemic GFLV agroinfection experiments employed treatments in which *A. tumefaciens* suspensions were co-cultured with acetosyringone (Vaghchhipawala *et al.*, 2010).

#### **GFLV accumulation and silencing suppressor efficacy experiments in infiltrated zones of *N. benthamiana***

GFLV genomes FF, FG, GF and GG were tested for virus accumulation against each other and an eGFP control at two, five and eight days post-inoculation (dpi) in *N. benthamiana*. Each treatment and time point utilized four plants and two leaves per plant (eight leaves per treatment). Within time points, plants (treatments) were randomized on a greenhouse bench. In a second experiment, infiltrated zones of FF, FG, GF, GG, FF<sub>2A</sub> and GF<sub>2A</sub> were tested for GFLV protein accumulation at nine dpi. Each treatment used four plants and two leaves per plant (eight leaves per treatment).

Silencing suppressors CMV 2b and TBSV p19 were tested in infiltrated zones of *N. benthamiana* against eGFP and RFP for effects on GFLV accumulation. Treatments were co-infiltrated with GFLV genomes FF, FG, GF or GG. Two leaves of seven plants per treatment per genome were agroinfiltrated and 11 to 13 leaf samples

were collected and tested by ELISA at seven dpi. Plants were randomized on a greenhouse bench and a third party researcher concealed all treatments until conclusion of the collection and data analysis.

### **GFLV protein and fluorescence measurements**

For each fluorescence or serological assay, two punches of a one-cm<sup>2</sup> cork borer (70 mg  $\pm$  8.2 mg) were collected and frozen until collections from each experiment were complete. Frozen samples were ground in 0.7 mL phosphate buffer pH 7.4 using a TissueLyser (Qiagen, Hilden, Germany) and 100  $\mu$ L of crude leaf extracts were applied to GFLV double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Bioreba, Reinach, Switzerland) in each of two wells (technical replicates) on 96 well plates containing positive and negative controls (Gottula *et al.*, 2013; Vigne *et al.*, 2013). Plates were read after one hour of substrate incubation. The Synergy2 96 well plate reader and Gen5 software package (Biotek Corporation, Winooski, VT) were used to measure absorbance, average reads of technical replicates and blank-subtract absorbance output of GFLV-negative *N. benthamiana* tissue. Materials and methods for quantitative eGFP measurements were described by Vigne *et al.* (2013).

### **Systemic GFLV infection experiments**

Fifteen experiments that iteratively tested variables in systemic infection were carried out over a one-year period. In 931 agroinfiltrated *N. benthamiana* plants, systemic GFLV infection was tested by ELISA at four weeks post-inoculation on

apical, non-inoculated leaves. The variables included genome identity (**Table 4-2**), silencing suppressor (TBSV p19 and CMV 2b) co-infiltration, acetosyringone co-culture, *A. tumefaciens* density ( $OD_{600nm}$  = 0.1 to 1.0), plant size (two versus six true leaves), and cooling degree days were estimated post-hoc. Several systemic infection experiments were not included in this analysis but were used to confirm the trends and are reported individually in the results.

### **Plant growth conditions and cooling degree day estimation**

*N. benthamiana* was grown in four-inch pots containing Cornell mix potting medium (Balaji *et al.*, 2011) in a greenhouse with a 16/8 hr. photoperiod maintained with lighting supplemented by high pressure sodium lamps. Plants were watered daily or every other day, as needed, and fertilized weekly. The greenhouses present at the Cornell University New York State Agricultural Experiment Station were low frame glasshouses with automatic temperature controls which were set to a constant temperature of 22°C. Greenhouse heating consisted of radiator and convection systems and cooling was achieved by evaporative coolers and automatic vents.

Since greenhouse temperatures were not directly recorded, estimates were obtained from historical weather data from a local weather station. Sum cooling degree days were calculated from a base temperature of 22°C for the four-week period of each plant systemic infection experiment (from date of inoculation to tissue collection). The degree day calculations were used as corollaries for ambient greenhouse temperatures.

### **Transformation of *N. benthamiana* and transgene insertion**

Constructs designed to express GFLV-F13 RNA1, GFLV-F13 RNA2, GFLV-GHu RNA1 and GFLV-GHu RNA2 were used to stably transform *N. benthamiana*. Clones in *A. tumefaciens* strain C58 were prepared as described for agroinfiltration experiments and applied to *N. benthamiana* leaf discs, as previously reported (Yepes *et al.*, 1996). The regenerated plants (R<sub>0</sub>) were tested by ELISA for expression of neomycin phosphotransferase II protein (nptII) (Agdia, Elkhart, IN) and by PCR for GFLV cDNAs insertion using primers G13 and G16 for RNA1 (Vigne *et al.*, 2013), and primers P1 and P2 for RNA2 (**Table 4-1**). R<sub>0</sub> plants were self-pollinated and T<sub>1</sub> plants were tested for transgene insertion and selectable marker expression by a kanamycin seed germination assay (seeds were germinated on MS medium with 300µg/mL kanamycin) and nptII ELISA. DNA extracted from T<sub>1</sub> plants were subjected to quantitative PCR using transgene primers P3 and P4 (**Table 4-1**) and reference (*β-actin*) primers (Quiapim *et al.*, 2009) with conditions and calculations according to Bubner *et al.* (2004).

One or two transgenic lines per GFLV construct showing segregation ratios consistent with *nptII* presence at one or two independent loci (qPCR) were used for functional transgene expression analysis, which included agroinfiltration of the complementary GFLV genome part and silencing suppressors followed by collection of an apical leaf at four weeks post-inoculation for determining GFLV infection by ELISA.

### **Agroinfiltration of *Nicotiana* spp.**

Five *Nicotiana* species (*N. benthamiana*, *N. clevelandii*, *N. glutinosa*, *N. obtusifolia*, and *N. paniculata*) were assayed for agroinfiltration expression capability (eGFP) and infiltrated-zone GFLV protein accumulation. Three plants and two leaves per plant were agroinfiltrated with the GFLV genome FG plus silencing suppressors (left side of each leaves) or eGFP plus silencing suppressors (right side of each leaf). The infiltrated zones were collected at five dpi and processed by ELISA for GFLV or fluorescence (eGFP) expression.

### **Statistics**

All statistics were conducted in SAS version 9.3 (SAS Institute, Cary, NC) unless otherwise noted. Each data set was verified for homogeneity of variance via Levene's tests ( $P > 0.05$ ) prior to ANOVA. Where Levene's test revealed significant deviation from homogeneity ( $P < 0.05$ ), non-parametric tests were conducted.

For the infiltrated zone experiment that compared agroinfiltrated-zone GFLV protein expression, absorbance outputs of individual GFLV genomes FF, FG, GF and GG were evaluated among time points by ANOVA, followed by Tukey post-hoc tests. A Kruskal-Wallis test, followed by Scheffe's test, was used to compare absorbance values of FF, FG, GF, GG, FF<sub>G2A</sub> and GF<sub>G2A</sub> using Excel Statistics Software 2010 (Social Survey Research Information Co., Ltd).

To compare relative GFLV ELISA OD<sub>405nm</sub> values and eGFP fluorescence (508<sub>nm</sub>), absorbance and fluorescence data points were divided by the maximum ELISA absorbance and eGFP fluorescence outputs, respectively, of each experiment

and multiplied by a factor of 100 to generate relative expression values. GFLV versus eGFP relative expression values were evaluated at each time point by the Dunnett's multiple range test. ELISA absorbance values for each GFLV genome were examined in the presence or absence of silencing suppressors and resulting data were subjected to Mann-Whitney-U test following  $\log^{10}$  transformation. An ANOVA followed by an LSD post-hoc test was used to compare GFLV accumulation.

Two binary logit regression models were used to assign odds ratios (ORs), 95% confidence intervals and *P* values to variables affecting systemic GFLV infection outcomes (Anderson *et al.*, 2003). For the first model, a binary logit regression in a stepwise method was employed to account for variables of GFLV infection including *A. tumefaciens* strain, plant size, the coinfiltration of versus absence of heterologous silencing suppressors, GFLV genome, and two continuous variables: density of *A. tumefaciens* and cooling degree days in the greenhouse for the four-week duration of the experiment. For the second analysis, a binary logit regression in direct method was used to account for differences in systemic GFLV infection depending on transgene versus agroinfiltration-delivery of a genome part. ORs were assigned based on genome or RNA2 delivery method (transgenic or transient expression).

## **RESULTS**

### **Creation and validation of GFLV constructs for agroinfiltration**

Full-length RNA1 and RNA2 cDNAs of GFLV strains F13 and GHu from previously validated transcription vectors (Viry *et al.*, 1993; Vigne *et al.*, 2013) were subcloned into CaMV 35S expression cassettes of pEPT8 and GFLV cDNAs in



CaMV 35S expression cassettes were cloned via PCR and restriction digestion into binary vector pGA482G for mobilization into *A. tumefaciens*. Complete sequencing of each clone revealed no polymorphisms between the original GFLV cDNA sequences and the corresponding cDNA sequences in binary vectors but GFLV GHu RNA1, GFLV GHu RNA2, and F<sub>G2A</sub> contained one CaMV 35S enhancer while all other clones contained a duplicated CaMV 35S enhancer. Each *A. tumefaciens* clone used in this study was verified to contain the appropriate binary vectors following plasmid extraction and restriction digestion.

#### **Effect of genome composition on GFLV accumulation in the agroinfiltrated zone of *N. benthamiana***

GFLV genomes FF, FG, GF and GG were agroinfiltrated into *N. benthamiana* leaves and virus accumulation was quantified (**Figure 4-1A**). There was a significant difference in ELISA absorbance output at two, five and eight dpi with *P* values of 0.0275, 0.0010 and 0.000, respectively according to ANOVA. FG produced higher expression than FF at two dpi, and FF and GF at five dpi. At eight dpi, FG showed greater expression than all other genomes, and GG expression exceeded that of FF and GF.

The experiment was repeated using FF, FG, GF, GG, FF<sub>G2A</sub> and GF<sub>G2A</sub> and samples were collected at nine dpi. The goal was to test whether protein 2A<sup>HP</sup> of strain GHu could be used to rescue the low GFLV accumulation associated with F13 RNA2 since protein 2A<sup>HP</sup> is involved in RNA2 replication (Gaire *et al.* 1999). Significant difference was detected in ELISA output among six GFLV genomes according to the

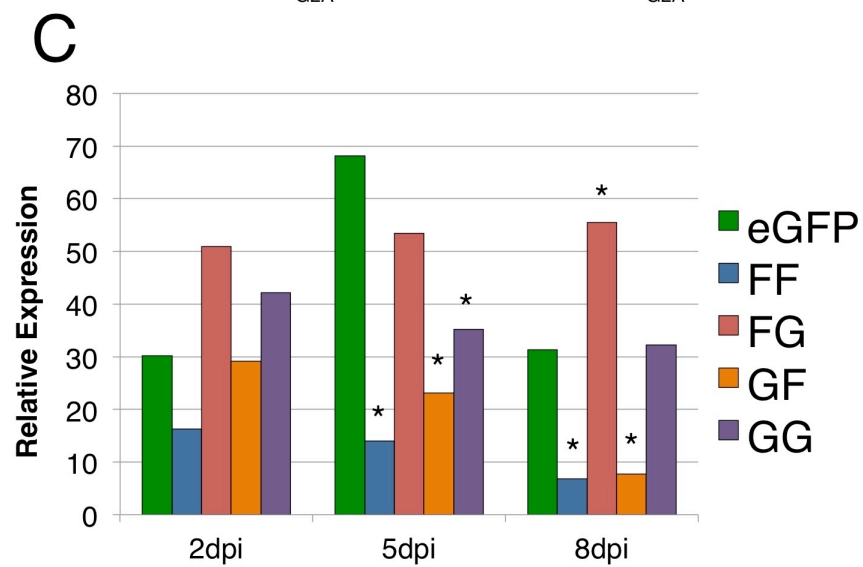
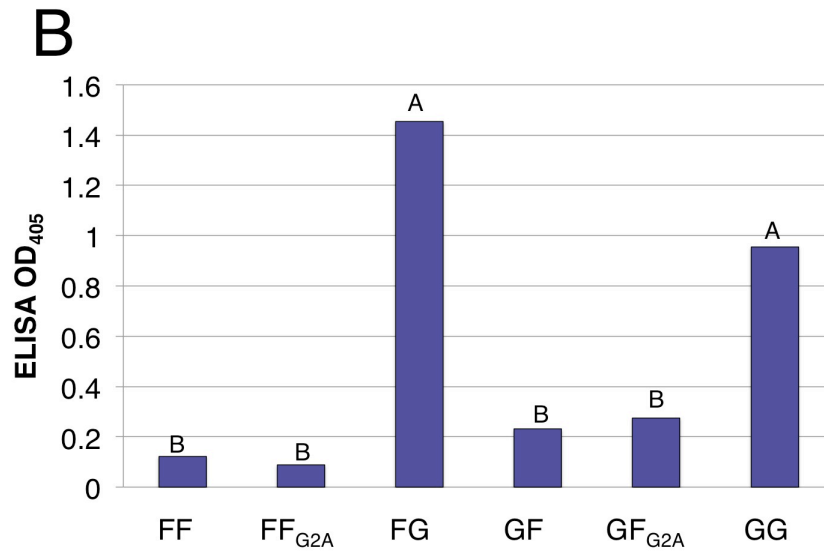
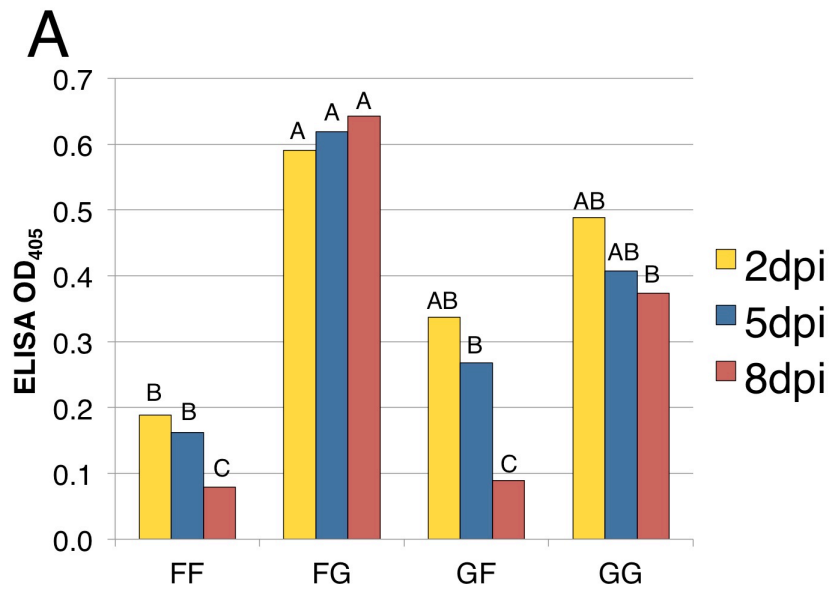
Kruskal-Wallis test ( $P=0.0001$ ). Accumulation of FF<sub>G2A</sub> or GF<sub>G2A</sub> following coinfiltration was not significantly different from FF or GF, and GG produced greater accumulation than FF and GF (**Figure 4-1B**). FG produced greater infiltrated zone accumulation than GG, and GG produced greater accumulation than FF and GF (**Figure 4-1B**). These results suggested that the composition of GFLV protein 2A<sup>HP</sup> does not impact GFLV expression levels in agroinfiltrated zones of *N. benthamiana*.

The ELISA outputs of each GFLV genome were compared to eGFP fluorescence expression at two, five and eight dpi (**Figure 4-1C**). As expected, eGFP showed a bell-curve expression pattern with a peak at five dpi. Although no individual GFLV genome showed a significant deviation from eGFP expression at two dpi according to Dunnett's test ( $P<0.05$ ), at five dpi, all genomes except FG were significantly lower than eGFP. At eight dpi, FG showed significantly higher accumulation than eGFP, and GF and FF showed significantly lower expression than eGFP. These data suggested that FG is able to rise above the expected level of *A. tumefaciens*-mediated protein expression and that protein expression of agroinfiltrated GF and FF is suppressed in plants compared to eGFP

### **Effect of silencing suppressors on GFLV protein expression**

GFLV genomes were tested for their response to coinfiltration of heterologous silencing suppressors. Silencing suppressors (CMV 2b and TBSV p19) or null controls (eGFP and RFP) were co-agroinfiltrated with the GFLV vectors and GFLV accumulation was measured by ELISA at seven dpi. Coinfiltration of silencing suppressors increased accumulation of each GFLV genome three to 23 fold, and to a

**Figure 4-1** Effect of *Grapevine fanleaf virus* genome composition on virus accumulation in agroinfiltrated zones of *Nicotiana benthamiana*. **A**, Accumulation of different GFLV genomes (see **Table 4-1** for details) was measured by ELISA at two, five and eight days post agroinfiltration (dpi). Same letters represent no significant difference for individual virus genomes among time points (n=8) at  $P<0.05$  according to Tukey. **B**, Accumulation of different GFLV genomes (See **Table 4-1** for details) measured by ELISA at nine dpi. Same letters represent no significant difference for individual virus genomes (n=8) at  $P<0.05$  according to Scheffe. **C**, Accumulation of different GFLV genomes against enhanced Green Fluorescent Protein (eGFP) at two, five and eight dpi. GFLV accumulation was measured by ELISA ( $A_{405nm}$ ) and eGFP fluorescence (508nm) using a Synergy2 microplate reader. Relative expression was calculated by converting measured values to percent maximum expression for each treatment. An asterisk (\*) indicates a treatment (n=8) was significantly less or greater than the eGFP control (n=8) at  $P<0.05$  at a given time point according to Dunnett's test.



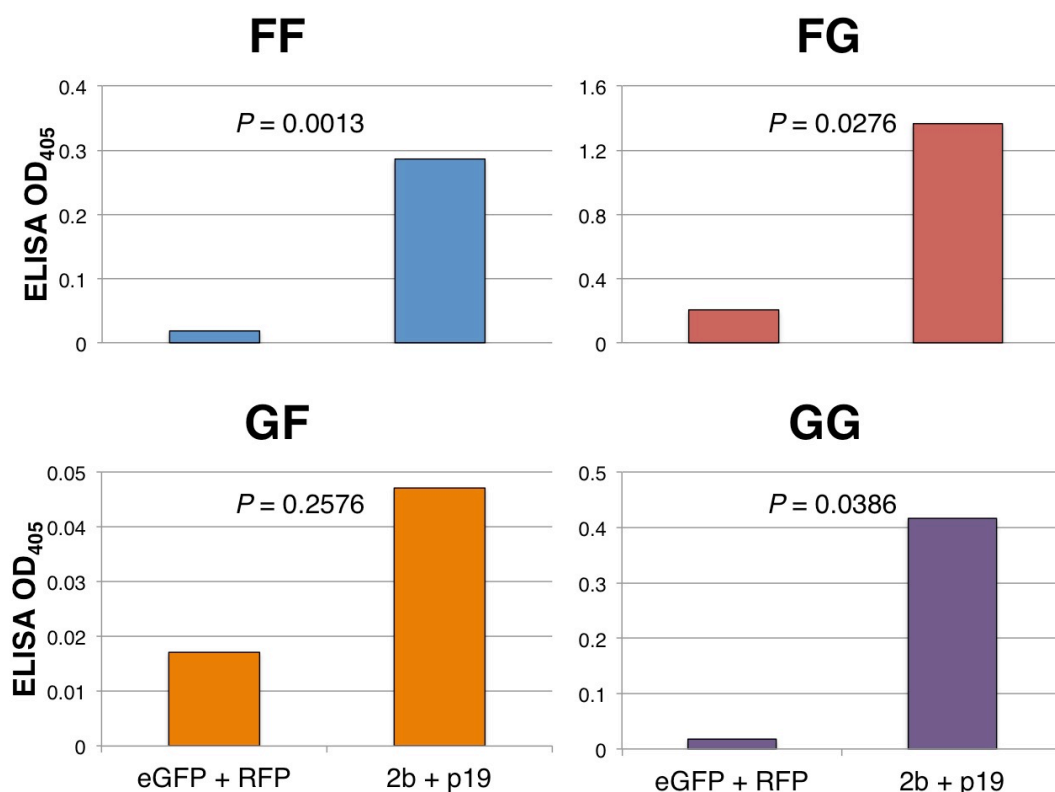
statistically significant level for FF, FG and GG (**Figure 4-2**). No significant difference was observed for GF, which showed the lowest virus accumulation in both treatments. These local infection analyses indicated that silencing suppressors can enhance GFLV accumulation in *N. benthamiana* following agroinoculation.

### **Systemic infection of the GFLV genomes**

Following observation of GFLV expression in agroinfiltrated zones, experiments were carried out to assess the frequency of systemic infection. The GFLV genomes tested were those used in infiltrated zone experiments and also included an additional GHu RNA2-based construct ( $G_R$ ), which was co-agroinfiltrated with F13 RNA1 ( $FG_R$ ) or GHu RNA1 ( $GG_R$ ) (**Table 4-2**).

In 15 independent experiments, 931 plants were agroinfiltrated with different GFLV genomes and exposed to other variables. A total of 239 plants achieved systemic infection. The systemic infection data were appropriate for stepwise logit regression analysis according to Hosmer and Lemeshow Goodness-of-Fit test ( $P=0.5782$ ). *A. tumefaciens* strain identity (LBA4404 or C58), co-culture with acetosyringone and plant size were not significant and these effects were excluded from the model. Other variables and interactions explained 90.4% of the systemic infection outcomes and included significant ( $P<0.05$ ) effects for genome composition, density of the *A. tumefaciens* suspension, cooling degree day accumulation and presence of silencing suppressors (**Table 4-3**).

The coinfiltration of silencing suppressors showed the largest effect of any variable considered. The binary logit model showed that silencing suppressors



**Figure 4-2** Effect of silencing suppressor 2b from *Cucumber mosaic virus* and p19 *Tomato bushy stunt virus* on *Grapevine fanleaf virus* accumulation in *Nicotiana benthamiana* following coinfiltration with genomes FF, FG, GF and GG. Heights of columns represent the mean (n=11 to 13) of ELISA absorbance values (OD<sub>405nm</sub>). Vectors expressing Red Fluorescent Protein (RFP) and enhanced Green Fluorescent Protein (eGFP) were used as controls. Displayed *P* values represent the significance of the difference between the two treatments calculated by Mann-Whitney U test.

**Table 4-3** Odds Ratio (OR) and 95% confidence intervals (CI) of variables in *Agrobacterium tumefaciens*-mediated GFLV infection of *Nicotiana benthamiana* based on a binary logit regression.

Variable	OR (95% CI)	P Value
<i>A. tumefaciens</i> density	0.251 (0.109-0.580)	0.0012
Cooling degree days	0.993 (0.991-0.995)	<.0001
Silencing suppressors (versus none)	12.811 (7.678-21.375)	<.0001
Genome FF <sub>G2A</sub> versus genome FF	6.807 (1.722-26.908)	0.0062
Genome FG versus genome FF	7.571 (3.589-15.975)	<.0001
Genome FG <sub>R</sub> versus genome FF	29.356 (3.427-251.454)	0.0020
Genome GF versus genome FF	0.281 (0.087-0.904)	0.0332
Genome GG versus genome FF	1.116 (0.450-2.772)	0.8123
Genome GG <sub>R</sub> versus genome FF	0.345 (0.082-1.462)	0.1487

increase the likelihood of infection by 12.8 fold (**Table 4-3**). Silencing suppressors positively affected frequencies of systemic infection in independent experiments as well. In one experiment, systemic infection occurred with FG and silencing suppressors in nine of 31 plants, whereas with FG without silencing suppressors (null controls) only in five of 31 plants. The effect on GG was more dramatic, where GG without silencing suppressors produced no infections in 31 plants and GG with silencing suppressors infected nine of 31 plants. The *A. tumefaciens* density had a negative effect on systemic infection where an OD<sub>600nm</sub> of 0.1 was more likely to produce systemic infection than an OD of 1.0 (**Table 4-3**).

Greenhouse temperatures fluctuated between 22°C and 32°C depending on ambient outdoor temperatures. Higher cooling degree days (hotter temperatures) had negative effects on the frequency of agroinfection (**Table 4-3**). This corresponded to observations that systemic infection was difficult to achieve in the low frame greenhouse in summer when temperatures regularly approached daytime highs of 32°C. In contrast, when temperatures remained close to the set 22°C constantly, systemic infection frequencies were improved.

The GFLV genome composition had a major effect on systemic infection. Maximum likelihood estimates indicate the performance of genomes relative to FF (**Table 4-3**). FF<sub>G2a</sub>, FG and FG<sub>R</sub> produced significantly more systemic infection than FF. Although GF<sub>G2A</sub> was included in this analysis, the sample size was too small to compute a reliable confidence interval and this genome did not produce a single instance of systemic infection in 18 plants in two independent experiments (data not shown). GG and GG<sub>R</sub> were not significantly different from FF ( $P < 0.05$ ) and GF



produced significantly less infection than FF (**Table 4-3**). The ORs and confidence intervals generated in the logit regression analysis reflect broader trends observed between genome composition and systemic infection. In one experiment that compared systemic infectivity of FG<sub>R</sub>, GG<sub>R</sub> to FG and GG each mixed with heterologous silencing suppressors at an *A. tumefaciens* suspension at OD<sub>600nm</sub>=1, FG<sub>R</sub> produced infection in 18 of 20 plants, GG<sub>R</sub> in 3 of 20 plants, FG in 10 of 10 plants and GG in 1 of 10 plants. These results showed that the composition of RNA1 and RNA2 affects systemic infection in *N. benthamiana* where F13 RNA1 outperforms GHu RNA1, and GHu RNA2 including the G<sub>R</sub> construct outperforms F13 RNA2.

### **Stable versus transient expression of GFLV constructs**

Transgenic T<sub>1</sub> *N. benthamiana* plants expressing F13 RNA1, GHu RNA1, F13 RNA2 or GHu RNA2 were generated. According to seed germination assays on selective medium, ELISA for nptII protein expression and qPCR-based transgene copy number estimation, T<sub>1</sub> lines segregated for one or two independent loci (data sets not shown). These tests allowed identification of null segregants, which were excluded from further analysis. Plants constitutively expressing RNA1 constructs were agroinfiltrated with GFLV GHu RNA2 and plants expressing RNA2 constructs were agroinfiltrated with GFLV GHu RNA1, and each *A. tumefaciens* culture containing a GFLV genome part was mixed with heterologous silencing suppressors. ELISA was used to test systemic GFLV infection at four weeks post-inoculation, respectively. Each transgenic line became systemically infected at least once in samples of 20 to 40 plants, which indicates stable full-length T-DNA integration in each line and

functional expression following complementation through agroinoculation. There results indicated that systemic infection can be achieved if a GFLV component is stably expressed and the other is transiently expressed.

A direct logit regression analysis was run to compare transgenic genome part expression versus agroinfiltrated genome expression on systemic infection outcomes. Out of 200 plants analyzed among five experiments, 47 plants became systemically infected. The data were appropriate for logit regression analysis as seen by a Hosmer-Lemeshow Goodness-of-Fit test ( $P=0.2054$ ). Transgenic expression of RNA1 and RNA2 penalized systemic infection with transgenic expression of RNA2 having the largest penalty. Systemic infection frequencies of genomes GG and FG were compared to GF when both genome parts were delivered by agroinfiltration versus one delivered by transgenic expression (**Table 4-4**). FG and GG performed better than GF in terms of capability to cause systemically infection if one genome part was delivered by transgene expression (**Table 4-4**). All constructs were penalized if one genome part was transgenically expressed: systemic infection was nine times less likely if RNA2 was stably rather than transiently expressed (**Table 4-4**). These results suggested that transgene expression of one genome part and agroinfiltration of the other genome part diminishes GFLV infection frequency compared to when both genome parts are agroinfiltrated.

### **Reaction of a *Nicotiana* panel to agroinfiltration with GFLV**

*N. benthamiana*, *N. clevelandii*, *N. paniculata*, *N. glutinosa* and *N. obtusifolia*

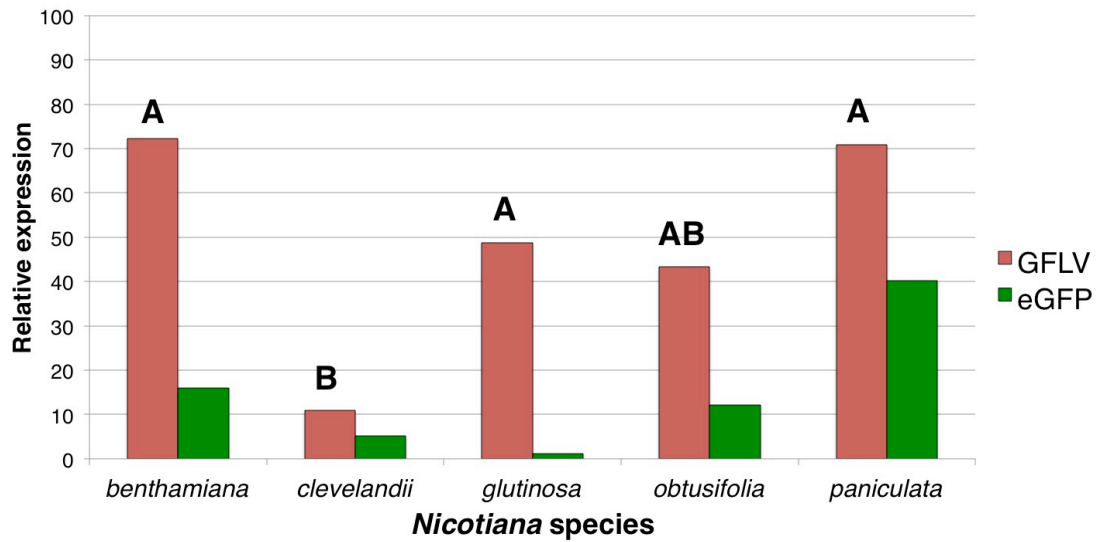
**Table 4-4** Odds Ratio (OR) and 95% confidence intervals (CI) of *Agrobacterium tumefaciens* versus transgenic expression of individual genome parts in *Nicotiana benthamiana* based on direct logistic regression.

<b>Variable</b>	<b>OR (95% CI)</b>	<b>P Value</b>
Genome FG versus genome GF	5.546 (0.684-44.981)	0.1087
Genome GG versus genome GF	10.566 (1.318-84.692)	0.0264
<i>A. tumefaciens</i> versus transgene delivery of RNA2	9.283 (2.540-33.931)	0.0008

were tested for GFLV accumulation following agroinfiltration with FG and fluorescence following eGFP agroinfiltration. Prior experiments had revealed various degrees of susceptibility to mechanical inoculation of GFLV strain GHu: *N. benthamiana* and *N. clevelandii* are completely susceptible (Vigne *et al.*, 2013), *N. obtusifolia* and *N. glutinosa* are highly resistant, and *N. paniculata* is moderately resistant, as shown by ELISA in non-inoculated apical leaves (Gottula *et al.*, 2014b). FG or eGFP were mixed with silencing suppressors 2b and p19 prior to infiltration. ELISA absorbance values representing GFLV protein accumulation were compared among plants species, and significant differences were obtained according to ANOVA ( $P=0.0057$ ). *N. benthamiana*, *N. paniculata* and *N. glutinosa* showed the highest GFLV accumulation followed by *N. obtusifolia* which showed intermediate GFLV expression, and *N. clevelandii* showed the lowest GFLV accumulation (**Figure 4-3**). Differences in eGFP expression could not explain the ELISA value differentials (**Figure 4-3**). These results indicate that GFLV protein accumulation following agroinfiltration does not reflect differences in resistance to mechanical inoculation in this *Nicotiana* panel, which suggest *Nicotiana* resistance to GFLV are altered by different inoculation methods, different GFLV genomes or co-application of heterologous silencing suppressors.

## ***DISCUSSION***

In this study, parameters for an optimal expression of CaMV 35S-driven RNA1 and RNA2 cDNAs from two distinct GFLV strains were determined following agroinfiltration in *N. benthamiana*. The GFLV system studied here has several



**Figure 4-3** Comparative expression of *Grapevine fanleaf virus* (GFLV) and eGFP among *Nicotiana benthamiana*, *N. clevelandii*, *N. glutinosa*, *N. obtusifolia* and *N. paniculata*. Two leaves per plant of three plants (n=6) were agroinfiltrated with GFLV-FG and 35S:eGFP, and ELISA and fluorescence measurements were taken at five days post-inoculation. GFLV ELISA absorbance values at 405nm and eGFP fluorescence emission values at 508nm were converted to relative expression values based on percent maximum expression for each measurement. Same letters represent no significant differences in GFLV expression in ELISA according to LSD (ANOVA,  $P<0.05$ ).

features that make it amenable to transient expression assays and evaluation of virus accumulation detection by ELISA. First, the GFLV antibodies (Bioreba) detect purified virus particles from strains F13 and GHu equally well (Vigne *et al.*, 2013). Second, the translation strategy of GFLV based on proteolytic processing of polyproteins means that ELISA-measured virus accumulation could be representative not only of the coat protein expression but also of the genome expression of the virus (Scholthof *et al.*, 1996). Third, GFLV cDNAs used in this study are identical to full-length cDNAs that produce functional *in vitro* transcripts: functionalities of *in vitro* transcripts FF, FG, GF, and GG were reported by Vigne *et al.* (2013), and functionalities of *in vitro* transcripts FG<sub>G2A</sub> and GF<sub>G2A</sub> were also verified (E. Vigne, unpublished results). These features allowed us to reliably test which factors impact GFLV agroinfection on local and systemic levels.

Almost 1,000 plants were tested for factors involved in systemic infection following agroinfection. *A. tumefaciens* strain identity [C58 (a nopaline strain) versus LBA4404 (an octopine strain)], presence or absence of acetosyringone and plant sizes were not identified as significant factors in systemic infection. Stepwise logit regression procedures attempt to parse effects of individual variables (risk factors) on a binary outcome (in this case, presence or absence systemic infection) but sometimes cannot separate the effect of one variable from another (Anderson *et al.*, 2003). Thus whether some variables tested in this study truly did not impact systemic infection remains unclear. However, the model did explain 90.4% of systemic infection outcomes, which suggested that the variables identified as significant (greenhouse temperatures, co-infiltration of silencing suppressors, genomes and *A. tumefaciens*

density) governed most of the successful systemic infection in these experiments.

Composition of the virus genome has major effects on successes and failures of diverse plant virus agroinfection systems (German-Retana *et al.*, 2003; Youssef *et al.*, 2011; Kurth *et al.*, 2012). By comparing the levels of GFLV accumulation to nonviral expression (35S:eGFP), autonomous viral expression levels could be distinguished from protein expression due to agroinfiltration with FG showing a stable or subtle increase in expression, GG a stable or slightly diminishing expression, and FF and GF a rapidly diminishing expression (**Figure 4-1C**). This indicated that GFLV-F13 RNA2 performed poorly and its weak performance could not be rescued by the 2A<sup>HP</sup> coding sequence of GFLV-GHu (**Figure 4-1B**). Compared to eGFP expression, FF and GF seem to be vulnerable to virus-specific suppression mechanisms. On the other hand, FG expression showed greater expression than eGFP at eight dpi, suggesting that the virus's autonomous replication outpaced antiviral defense mechanisms in *N. benthamiana*. The flat-line trajectory of GG between two, five and eight dpi suggested this genome maintains equilibrium between defense and replication.

The systemic infection frequencies of individual genomes were not always related to their accumulation capabilities in infiltrated leaves. FF<sub>G2A</sub> outperformed FF in terms of systemic infection but not local infection, and conversely, GFLV protein of GG accumulated at higher levels than FF, but did not show significantly higher systemic infection frequencies. These discrepancies suggest that factors governing local infection are different from those that influence systemic infection. The differences between local and systemic infection could relate to the transcript's RNA

structure (Meshcheriakova *et al.*, 2014) or to systemic GFLV movement, which is determined by its movement and coat proteins (Andret-Link *et al.*, 2004).

Silencing suppressors enhance agroinfection of diverse viruses (Chiba *et al.*, 2006; Wege and Siegmund, 2007; Yoon *et al.*, 2011). Therefore, the effect of silencing suppressor coinfiltrated with GFLV genomes on FF, FG, GF and GG accumulation was tested in patch and systemic infection assays. The silencing suppressors CMV 2b and TBSV p19 together increased FF, FG and GG accumulation to statistically significant levels (**Figure 4-2**). Notably, silencing suppressors did not equalize the genomes' overall expression levels: with or without silencing suppressors the pattern of infiltrated zone virus accumulation was superior for the FG genome. This suggested that RNA silencing is an important factor that dampens viral expression following agroinfection, but may not be the sole factor. Antiviral mechanisms specific to *A. tumefaciens*-mediated delivery may operate particularly strongly against F13 RNA2 constructs including the 2A<sup>HP</sup> recombinant construct because the corresponding constructs resulting from *in vitro* transcription are perfectly functional *in planta* (Amari *et al.*, 2010; Vigne *et al.*, 2013).

The G<sub>R</sub> construct produced similar systemic infection frequencies compared to the analogous GHu RNA2 construct. G<sub>R</sub> is comprised of a GHu RNA2 expression cassette with a separate CMV 2b expression cassette on the same T-DNA (Gottula *et al.*, 2014a). The association of high GHu RNA2 systemic infection when a silencing suppressor is co-expressed from the same T-DNA corresponds well to the positive correlation between coinfiltration of silencing suppressors and systemic infection. This could suggest that the delivery of a silencing suppressor *in trans* is equally as effective



as its delivery *in cis*. The FG<sub>R</sub> genome produced nearly 100% infection in *N. benthamiana* and formed the basis of GFLV heterologous gene delivery, reported elsewhere (Gottula *et al.*, 2014a).

The cloning procedure produced several constructs with a single rather than a double CaMV 35S enhancer sequence (GHu RNA1, GHu RNA2 and the F<sub>G2A</sub> constructs). The truncation of the CaMV 35S promoter sequence was likely due to homology of the PCR primers with the duplicated enhancer sequences. The number of CaMV 35S enhancers could affect the level of viral RNA expression. However, since GHu RNA2 was the best performing RNA2 construct and GHu RNA1 was the worst performing RNA1 construct in terms of local and systemic infection of *N. benthamiana*, single versus double CaMV enhancers does not solely account for discrepancies in agroinfection efficacies.

Transgenic *N. benthamiana* plants that express the full-length cDNAs of RNA1 or RNA2 of GFLV strains F13 and GHu were used to test whether stable integration and constitutive expression of the T-DNAs could enhance the infection potential when the complement constructs were agroinfiltrated. Because the T-DNAs containing RNA1 cDNAs exceed 10kb, it was hypothesized that *A. tumefaciens* does not consistently integrate the full-length genome during transient expression and that could explain the lower-than-expected infection frequency. On the contrary, stable integration of the RNA1 or RNA2 T-DNAs decreased the infection potential compared to transient expression experiments in which both T-DNAs were agroinfiltrated. Calvo *et al.* (2010) found that stably integrated *Plum pox virus* cDNA in *N. benthamiana* rarely converts into infective viral transcripts and that genome

expression is suppressed by gene silencing. Transgenically expressed RNA viral genomes may fail to efficiently infect due to unfavorable timing or level of genome expression relative to the onset of resistance mechanisms. .

Greenhouse temperature and density of *A. tumefaciens* suspension were negatively correlated to systemic infection outcomes (**Table 4-4**). Higher temperatures may negatively impact virus replication following agroinfiltration (Jiang *et al.*, 2011) and do negatively affect nepovirus systemic infection following mechanical inoculation (Siddiqui *et al.*, 2008). The negative relationship of *A. tumefaciens* density on systemic infection was surprising given that higher concentrations of *A. tumefaciens* can promote greater heterologous viral protein expression (Lindbo, 2007). This could indicate that there is a fine balance between GFLV protein expression following agroinfiltration, and activation of host defenses that target GFLV RNAs

One potential use of the GFLV constructs is to test plants for GFLV resistance following agroinfiltration (Bhaskar *et al.*, 2009). To determine if GFLV agroinfiltration provide a reliable inoculation method to screen for resistance, five *Nicotiana* species with varying degrees of resistance and susceptibility to mechanical inoculation (Gottula *et al.*, 2014b) were agroinfiltrated with the GFLV genome FG and silencing suppressors. No relationship was obtained between pre-defined GFLV resistance status and virus accumulation in agroinfiltrated leaves even when *A. tumefaciens* transient expression for eGFP was taken into account. For example, the susceptible *N. clevelandii* showed lower GFLV accumulation in agroinfiltrated zones than the resistant *N. glutinosa* even though *N. clevelandii* produced more eGFP fluorescence on average. These results suggested that host identity plays a major role

in infiltrated zone GFLV accumulation and is not related to GFLV susceptibility following mechanical inoculation. This discrepancy could be explained by the fact that silencing suppressors were coinfiltrated with GFLV in these experiments, which could mask important differential innate resistance mechanisms in these hosts. Another explanation could be the different GFLV genome composition (GFLV-GHu in mechanical inoculation experiments and FG in transient expression experiments) with regard to resistance/virulence interactions. It is known that host identity can play a role in agroinfection (Satyanarayana *et al.*, 2001; German-Retana *et al.*, 2003), but the mechanisms and significance of these differences observed with GFLV are unclear.

Unlike mechanical or insect vector-based inoculation strategies, agroinoculation of RNA viruses can present artificial constraints including *A. tumefaciens* pathogenesis, efficiency of faithful T-DNA transfer, nuclear transcription, processing and export of viral RNAs, non-specific transcriptional start sites from plant promoters, the presence of cryptic introns within viral cDNAs and altered host resistance dynamics. Future GFLV vectors could potentially benefit from more prudent vector designs including the use of promoters with specific transcriptional start sites, which has shown to boost agroinoculation efficacy of *Alternanthera mosaic virus* (Lim *et al.*, 2010) and *Tobacco mosaic virus* (TMV) (Komarova *et al.*, 2012a). TMV vector cDNAs showed improved infectivity if cryptic introns were removed or replaced by heterologous intron sequences (Marillonnet *et al.*, 2005; Komarova *et al.*, 2012b). The co-dependence of the GFLV genome parts on systemic plant infection could warrant the co-delivery of RNA1 and RNA2 components on a single T-DNA. Single cell co-delivery of RNA1 and RNA2 could be achieved by using binary vectors

specialized in transformation of very large T-DNA inserts (BiBACs) (Hamilton *et al.*, 1999). Using a BiBAC vector was associated with markedly improved agroinfection of the very large viral cDNA insert (about 19kb) comprising the *Citrus tristeza virus* vector (Ambros *et al.*, 2011). Nevertheless, this research has defined variables necessary for success of GFLV agroinfection in *N. benthamiana* such as coinfiltration of silencing suppressors, low temperature and GFLV genome composition, and has raised new questions about GFLV resistance dynamics in *Nicotiana* species.

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## CHAPTER 5

### ***TOWARDS A QUARTER CENTURY OF PATHOGEN-DERIVED RESISTANCE AND PRACTICAL APPROACHES TO PLANT VIRUS DISEASE CONTROL***

#### ***ABSTRACT***

The concept of pathogen-derived resistance (PDR) describes the use of genetic elements from a pathogen's own genome to confer resistance in an otherwise susceptible host via genetic engineering [J. Theor. Biol. 113 (1985) 395]. Illustrated with the bacteriophage Q $\beta$  in *Escherichia coli*, this strategy was conceived as a broadly applicable approach to engineer resistance against pathogens. For plant viruses, the concept of PDR was validated with the creation of tobacco plants expressing the coat protein gene of *Tobacco mosaic virus* (TMV) and exhibiting resistance to infection by TMV [Science 232 (1986) 738]. Subsequently, virus-resistant horticultural crops were developed through the expression of viral gene constructs. Among the numerous transgenic crops produced and evaluated in the field, papaya resistant to *Papaya ringspot virus* (PRSV) [Annu. Rev. Phytopathol. 36 (1998) 415] and summer squash resistant to *Cucumber mosaic virus* (CMV), *Zucchini yellow*

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*mosaic virus*, and/or *Watermelon mosaic virus* [Biotechnology 13 (1995) 1458] were released for commercial use in the USA. Although cultivated on limited areas, the adoption rate of cultivars derived from these two crops is increasing steadily. Tomato and sweet pepper resistant to CMV and papaya resistant to PRSV were also released in the People's Republic of China. Applying the concept of PDR provides unique opportunities for developing virus-resistant crops and implementing efficient and environmentally sound management approaches to mitigate the impact of virus diseases. Based on the tremendous progress made during the past quarter century, the prospects of further advancing this innovative technology for practical control of virus diseases are very promising.

## **INTRODUCTION**

Plant viruses are responsible for severe economic crop losses worldwide (Hull, 2002). The development and use of resistant crop cultivars is the most efficient strategy to mitigate the impact of virus diseases in agricultural settings. Traditionally, host resistance is exploited by conventional breeding methods to create virus-resistant cultivars. Protection from virus infection can be achieved by using dominant or recessive genes. Examples of dominant resistance genes are *Ry* for *Potato virus Y* (PVY) in potato and *Sw5* for *Tomato spotted wilt virus* (TSWV) in tomato. The *eukaryotic translation initiation factor* (*eIF4E*) is an example of a recessive resistance gene for potyviruses (Kang *et al.*, 2005; Lanfermeijer and Hille, 2007).

The concept of pathogen-derived resistance (PDR) offers a different approach to develop virus-resistant crop plants. This concept was conceived a quarter century ago (Sanford and Johnston, 1985). It describes the engineering of resistance in otherwise susceptible hosts, including plants, by using genetic elements, for example coding and noncoding sequence elements, from a pathogen's own genome (Sanford and Johnston, 1985). Cross-protection, a biological means for protecting plants from virus infection, was considered as an example of the concept of PDR that is already operational in nature. Cross-protection relies on the use of mild virus strains to protect plants from economic damage caused by severe virus strains (Fuchs *et al.*, 1997; Muller and Rezende, 2004). It was argued that a mutated form of a viral replicase similar enough to the one encoded by a challenge virus could bind to cell host attachment sites and prevent virus replication (Sanford and Johnston, 1985).

For plant viruses, the concept of PDR was first validated with the development



of tobacco expressing the coat protein gene of *Tobacco mosaic virus* (TMV) and exhibiting resistance to TMV infection (Powell Abel *et al.*, 1986). This breakthrough discovery paved the way for the creation of numerous virus-resistant transgenic plants, including horticultural crops. Some crop plants expressing viral genetic elements have been tested successfully in the field and a few have been commercialized. The deployment of virus-resistant transgenic plants has become an important strategy for effective and sustainable control of major virus diseases. This chapter provides a historical perspective on the concept of PDR from its inception to the release of the first virus-resistant transgenic crop resulting from its application. It also discusses how this concept led to an explosion in the development of virus-resistant plants and discusses advances made in terms of practical control of virus diseases during the past 25 years.

## ***THE CONCEPT OF PDR***

### **A description of the concept of PDR**

The concept of PDR describes the use of a pathogen's own genetic material as resistance genes for engineering resistance in an otherwise susceptible host. Sanford and Johnston, the two visionary scientists who articulated the concept, initially proposed this strategy as a broadly applicable approach for genetically engineering resistance to parasites. Resistance was hypothesized to be routinely achievable by cloning appropriate parasite genes, modifying their expression, if necessary, and transferring them into the host genome.

Sanford and Johnston (1985) reasoned that pathogens produce molecules that

are unique and critical for their pathogenic process. They proposed that dysfunctional pathogen-derived gene products could inhibit the pathogen by disrupting pathogen–host interactions if expressed by a host cell genome. To this extent, resistance could theoretically be achieved from the pathogen’s own genetic material. The predicted advantages of this approach to engineer resistance in an otherwise susceptible host were that (i) genes from a pathogen would have a minimal effect on the host and likely not produce substances harmful to humans, (ii) the resistance was anticipated to be more stable than host resistance, (iii) cloning genes from a pathogen would be relatively easy compared to host genes due to the small genome size, and (iv) genes from a pathogen would be always present and available for cloning purposes regardless of the diversity of the pathogen. For plant viruses, the concept of PDR and successful transfer of foreign DNA into plant cells that regenerate into transgenic plants opened new avenues for the development of virus-resistant plants.

### **A conceivable application of the concept of PDR**

Sanford and Johnston (1985) used the bacteriophage Q $\beta$  as a model to illustrate the concept of PDR. They hypothesized that four Q $\beta$ -encoded gene constructs, for example the coat protein gene, a modified replicase gene, the RNA segment encoding the replicase binding site, and the gene encoding the maturation protein, as well as an antisense RNA complementary to the Q $\beta$  RNA could be used as resistance genes against the bacteriophage Q $\beta$  in *Escherichia coli*. Sanford and Johnston (1985) further suggested that the strategy outlined for the bacteriophage Q $\beta$  in *Escherichia coli* could have a broader application for engineering resistance to other pathogens, opening an

unsuspected path for practical control of diseases. To this extent, the stage was set for innovative ways to create virus-resistant plants by applying the concept of PDR and developing efficient protocols for plant transformation.

## ***HISTORICAL PERSPECTIVES***

### **The first application of the concept of PDR for virus resistance in a model host**

Powell Abel *et al.* (1986) were the first to apply the concept of PDR to a plant virus. These authors produced *Nicotiana tabacum* cv. Xanthi and cv. Samsun expressing the coat protein gene of TMV and showed that transgenic tobacco exhibited resistance following infection by TMV via mechanical inoculation. Some transgenic tobacco failed to express symptoms for the duration of the experiments whereas others exhibited a substantial delay (two to 14 days) in disease development. Resistance was related to the level of expression of the viral coat protein and could be overcome by high doses of inoculum under which conditions plants developed typical systemic symptoms and systemically infected leaves contained high TMV titer (Powell Abel *et al.*, 1986). Plants had only a slight enhanced resistance to TMV RNA as inoculum (Nelson *et al.*, 1987). The resistance was strong to tobamoviruses closely related to TMV but weak or not detectable to distantly related tobamoviruses (Nejdat and Beachy, 1990). Additional experiments suggested that increased levels of TMV coat protein expression correlated with increased levels of resistance (Osbourn *et al.*, 1989; Powell Abel *et al.*, 1989; Prins *et al.*, 2008; Register and Beachy, 1988, 1989). The initial intent of Beachy and colleagues for transferring and expressing the TMV coat protein gene into tobacco was to gain a better understanding of the mechanisms

of cross-protection and provide new insights into virus–host interactions. Their seminal work launched a new era for the production of virus-resistant plants.

### **Other early applications of the concept of PDR for virus resistance**

As a consequence of the discovery by Powell Abel *et al.* (1986), resistance to numerous plant viruses was engineered primarily by using coat protein genes (Beachy *et al.*, 1990; Prins *et al.*, 2008; Tepfer, 2002). Other viral sequences, such as the RNA-dependent RNA polymerase read-through domain of TMV were also shown to induce resistance (Golembowski *et al.*, 1990), as well as the movement protein (Malysenko *et al.*, 1993), proteinase (Maiti *et al.*, 1993; Vardi *et al.*, 1993), satellite RNA (Gerlach *et al.*, 1987; Harrison *et al.*, 1987), defective interfering RNA (Kollar *et al.*, 1993), and 5' (Nelson *et al.*, 1993; Stanley *et al.*, 1990) and 3' (Zaccomer *et al.*, 1993) noncoding regions. It soon became apparent that almost any viral genetic element could be used to confer resistance to virus infection in plants. These observations validated some of the earlier predictions by Sanford and Johnston (1985) on the notion that several genes from a pathogen could be used to engineer resistance.

### **The concept of PDR and the antiviral pathways of RNA silencing**

The mechanism of engineered resistance through the application of the concept of PDR was poorly understood 25 years ago. It was hypothesized that a dysfunctional viral gene in a host could somehow interfere with virus multiplication. By analogy with cross-protection, the mechanisms consisted conceivably of competition for host factors, inhibition of the uncoating of challenge virus (Sherwood, 1987), disruption of

the replication of the challenge virus due to annealing of RNA species of the protective and challenge viruses (Palukaitis and Zaitlin, 1984), among other plausible explanations.

Expression of a viral coat protein in a transgenic plant was suggested initially to interfere with the uncoating step during an early event of the virus multiplication cycle (Osbourn *et al.*, 1989; Register and Beachy, 1988). Interaction of the viral coat protein with a host component or directly with the challenge viral RNA was hypothesized to prevent replication, translation, or virion assembly (Asurmendi *et al.*, 2007; Beachy, 1997, 1999; Bendahmane and Beachy, 1999; Clark *et al.*, 1995). Subsequently, a breakthrough discovery showed that an untranslatable coat protein gene of *Tobacco etch virus* (TEV) protected tobacco plants from TEV infection. Resistant plants were immune to TEV infection (Lindbo and Dougherty, 1992a, b). A recovery phenotype was also observed with plants infected and displaying symptoms similar to those of nontransgenic plants but newly emerging leaves were asymptomatic two weeks post-inoculation and transgene mRNA as well as viral RNA were rapidly degraded (Lindbo *et al.*, 1993). It became clear that the viral transgene protein product was not needed for engineered resistance and that there was an inverse correlation between transgene expression and resistance to virus infection (Dougherty *et al.*, 1994). In other words, the TEV coat protein RNA sequence was responsible for the resistance phenotype rather than the coat protein itself. This was unexpected as it was suggested that plants expressing high levels of viral coat protein would be likely resistant to virus infection in comparison with plants expressing little or no viral coat protein (Lindbo and Dougherty, 2005). Similar findings were published early on for

TSWV (de Haan *et al.*, 1992) and *Potato virus X* (PVX) (Longstaff *et al.*, 1993) in tobacco plants. While coat protein-mediated resistance is effective against a number of viruses (Asurmendi *et al.*, 2007; Bendahmane and Beachy, 1999; Dinant *et al.*, 1998; Schubert *et al.*, 2004; Wintermantel and Zaitlin, 2000), the majority of PDR phenomena seem to work through RNA-mediated mechanisms (Baulcombe, 2007; Eamens *et al.*, 2008; Prins *et al.*, 2008; Voinnet, 2008).

Plant RNA-dependent RNA polymerase and double-stranded (ds) RNAase activities were proposed to be part of the mechanism of resistance by producing short RNA of 10–20 nt in length complementary in sequence to the RNA to be degraded from the transgene RNAs (Lindbo *et al.*, 1993). These short RNAs would target specific RNAs for degradation by a dsRNase activity (Dougherty and Parks, 1995). The studies by Dougherty and colleagues advanced our understanding of the mechanisms underlying engineered virus resistance in plants and highlighted the role of a sequence-specific RNA degradation phenomenon through post-transcriptional gene silencing (PTGS). Their findings paved the way to the discovery of RNA silencing as a potent defense mechanism against plant viruses (Baulcombe, 2004, 2007; Eamens *et al.*, 2008; Lin *et al.*, 2007; Prins *et al.*, 2008; Voinnet, 2001, 2005, 2008; Waterhouse *et al.*, 1999, 2001). Later, it was shown that antiviral silencing occurred during the recovery phase of virus infection in nontransgenic plants (Covey *et al.*, 1997; Ratcliff *et al.*, 1997).

RNA silencing is initiated by double-stranded RNA (dsRNA) structures that are identical to the RNA to be degraded (Waterhouse *et al.*, 1998). Silencing is associated with the production of 21–25 nt duplexes called small interfering RNAs

(siRNAs) (Hamilton and Baulcombe, 1999; Hamilton *et al.*, 2002). The siRNAs are produced from dsRNA precursors by an endonuclease known as Dicer and become incorporated and converted to single-stranded RNAs (ssRNAs) in a Argonaute-containing ribonuclease complex (RISC) that target RNA for cleavage (Deleris *et al.*, 2006; Hannon, 2002; Obbard *et al.*, 2009; Voinnet, 2001, 2005, 2008). The pioneering work by Baulcombe and Waterhouse and their respective colleagues showed that RNA silencing is an innate and potent plant response to virus infection and a natural example of the concept of PDR.

### **The first application of the concept of PDR to a horticultural crop**

Soon after its first application for virus resistance in a model host plant (Powell Abel *et al.*, 1986), the concept of PDR was validated in a horticultural crop with the aim of providing practical control of a viral disease. Tomato was the first horticultural crop engineered for virus resistance through the application of the concept of PDR. In the first field trial ever of transgenic plants engineered for virus resistance, tomato plants expressing the coat protein gene of TMV were evaluated for resistance to mechanical inoculation by TMV (Nelson *et al.*, 1988). Only 5% of the transgenic plants were symptomatic at the end of the trial compared with 99% of the nontransformed control plants. Also, inoculated transgenic and uninoculated nontransformed plants had identical fruit yield, indicating that the transformation process and expression of the TMV coat protein gene did not alter the horticultural performance of the transgenic tomato plants. Sanders *et al.* (1992) extended the field characterization of transgenic tomato plants and showed resistance to distinct strains

of TMV. These studies confirmed Sanford and Johnston's conception of PDR as a practical solution for controlling virus diseases in plants.

## ***CREATION OF VIRUS-RESISTANT TRANSGENIC CROPS BY APPLYING THE CONCEPT OF PDR***

### **Early applications**

Effective resistance is desirable against virus inoculation via vectors to manage, for instance, aphid-transmitted virus diseases. The efficiency of viral genes at conferring resistance against vector-mediated virus transmission was shown first with cucumber plants engineered for resistance to *Cucumber mosaic virus* (CMV).

Cucumber plants expressing the coat protein gene of CMV had a significantly reduced incidence of CMV and a lower percentage of symptomatic plants than nontransformed control plants following CMV inoculation via aphid vectors (Gonsalves *et al.*, 1992).

In these studies, mechanically inoculated cucumber plants dispersed throughout the field provided reliable sources of inoculum for natural aphid populations to vector CMV. This approach coupled with the fact that field trials were established at a time of abundant endemic aphid flights caused sufficient disease pressure to make inferences about disease progress, resistance, and yield (Gonsalves *et al.*, 1992).

Subsequently, many other studies have illustrated the usefulness of engineered resistance at providing practical control of aphid-transmitted virus diseases (reviewed by Fuchs and Gonsalves, 2007).

Resistance to more than one virus is useful for practical control of virus diseases as mixed virus infections are common in agricultural settings. PDR offers



unique solutions to mixed virus infection, for example, by co-engineering and co-transferring genes from several viruses into a single host plant. The usefulness of multiple viral genes to control mixed virus infections was demonstrated early on with potato plants expressing the coat protein genes of PVX and PVY (Kaniewski *et al.*, 1990; Lawson *et al.*, 1990). Potato line 303 was highly resistant to infections by PVX and PVY in the field (Kaniewski *et al.*, 1990). Later, summer squash plants expressing coat protein gene constructs of CMV, *Zucchini yellow mosaic virus* (ZYMV), and/or *Watermelon mosaic virus* (WMV) were engineered for resistance to single viruses and combinations of these three viruses (Tricoli *et al.*, 1995). Among summer squash engineered for multiple virus resistance, line ZW-20 expressing the coat protein genes of ZYMV, and WMV was highly resistant whether infection occurred by mechanical inoculation or was mediated by aphid vectors (Fuchs and Gonsalves, 1995; Tricoli *et al.*, 1995). In addition, line CZW-3 expressing the coat protein genes of CMV, ZYMV and WMV was highly resistance to mixed infections by these three viruses (Fuchs *et al.*, 1998; Tricoli *et al.*, 1995). The three coat protein genes used to engineer multiple virus resistance in summer squash were transferred successfully in cantaloupes (Fuchs *et al.*, 1997). The concept of PDR has provided a platform for virus control that has facilitated new approaches to develop resistant crop cultivars and expanded opportunities to implement effective and sustainable management strategies of virus diseases.

### **Other examples**

Agronomic and horticultural plants, such as cereal, vegetable, legume, flower,

forage, turf, and fruit crops expressing virus-derived gene constructs have been created (Fuchs and Gonsalves, 2007). While testing in the field is underway for at least one dozen crop species expressing sequences derived from numerous viruses, very few of these field trials have yet been published in scientific journals.

Part of the difficulty of field-testing for resistance evaluation is ensuring high and consistent virus inoculation that can distinguish resistant and susceptible phenotypes (Gilbert *et al.*, 2009). Inoculation from external field sources can be reliable when studies are with insect-transmitted viruses. Presence of naturally viruliferous aphid populations allowed Lee *et al.* (2009) to discern resistant pepper expressing a CMV coat protein gene in conditions relevant to commercial agriculture. Natural infection by thrips vectors yielded statistically significant differences in TSWV incidence in peanut expressing an antisense TSWV nucleoprotein sequence (Magbanua *et al.*, 2000). In another study, peanut expressing a TSWV nucleoprotein had a strong tendency to be asymptomatic under field locations, although resistance was moderate following mechanical inoculation in a growth chamber (Yang *et al.*, 2004). Transgenic peanut and pepper showed good yield and quality parameters, respectively (Lee *et al.*, 2009; Yang *et al.*, 2004). For perennial crops, plum trees expressing a coat protein gene construct of *Plum pox virus* (PPV) were highly resistant to PPV infection during 6–8 years in varied orchard locations in Europe (Capote *et al.*, 2007; Hily *et al.*, 2004; Malinowski *et al.*, 2006; Ravelonandro, 2007; Zagrai *et al.*, 2008). The growth of knowledge about RNA silencing has provided a basis to optimize constructs to make engineered resistance to viruses more reliable and broadly applicable.

### More recent applications

The trigger for RNA silencing is dsRNA or double-stranded regions within the secondary structure of single-stranded RNA (Eamens *et al.*, 2008; Prins *et al.*, 2008; Voinnet, 2008; Waterhouse *et al.*, 1998). Several approaches have been used to express dsRNA cognate to viral RNA for activation of RNA silencing. Expressing sense and antisense viral genes or inverted repeat viral genes to express hairpin RNAs (hpRNA) for the formation of duplex RNA are some of the most recent strategies to engineer resistance (Missiou *et al.*, 2004; Praveen *et al.*, 2009; Prins *et al.*, 2008; Smith *et al.*, 2000; Tougou *et al.*, 2006; Wesley *et al.*, 2001). For example, intron-spliced hairpin RNA (ihpRNA), ihpRNA overhang, and ihpRNA spacer were evaluated for resistance to PVY (Smith *et al.*, 2000; Wesley *et al.*, 2001). The ihpRNA was found to be the most efficient constructs to conferring resistance to PVY with 90% of the plants exhibiting RNA silencing (Wesley *et al.*, 2001). The same strategy based on the use of highly conserved genetic segments of several viruses into a single transgene construct achieved multiple virus resistance (Bucher *et al.*, 1996).

Artificial plant micro RNA (amiRNAs) can also be used for virus resistance. The *Arabidopsis thaliana* pre-miR159a precursor was used to generate two amiRNAs<sup>159</sup> (amiR-P69<sup>159</sup> and amiR-HC-Pro<sup>159</sup>) with sequences complementary to *Turnip yellow mosaic virus* (TYMV) and *Turnip mosaic virus* (TuMV), respectively (Niu *et al.*, 2006). The amiR- P69<sup>159</sup> was designed to target the TYMV silencing suppressor P69 while amiR-HC-Pro<sup>159</sup> targeted the TuMV silencing suppressor HC-Pro. Transgenic plants carrying both transgenes expressed the corresponding amiRNAs and showed specific resistance to TYMV and TuMV. Low temperatures

had no substantial effect on miRNA accumulation (Niu *et al.*, 2006). Similarly, the miR171 of *Nicotiana benthamiana* was used to target the 2b gene of CMV and confer resistance to CMV (Qu *et al.*, 2008).

## ***COMMERCIALIZATION OF VIRUS-RESISTANT TRANSGENIC CROPS AND PRACTICAL CONTROL OF VIRUS DISEASES***

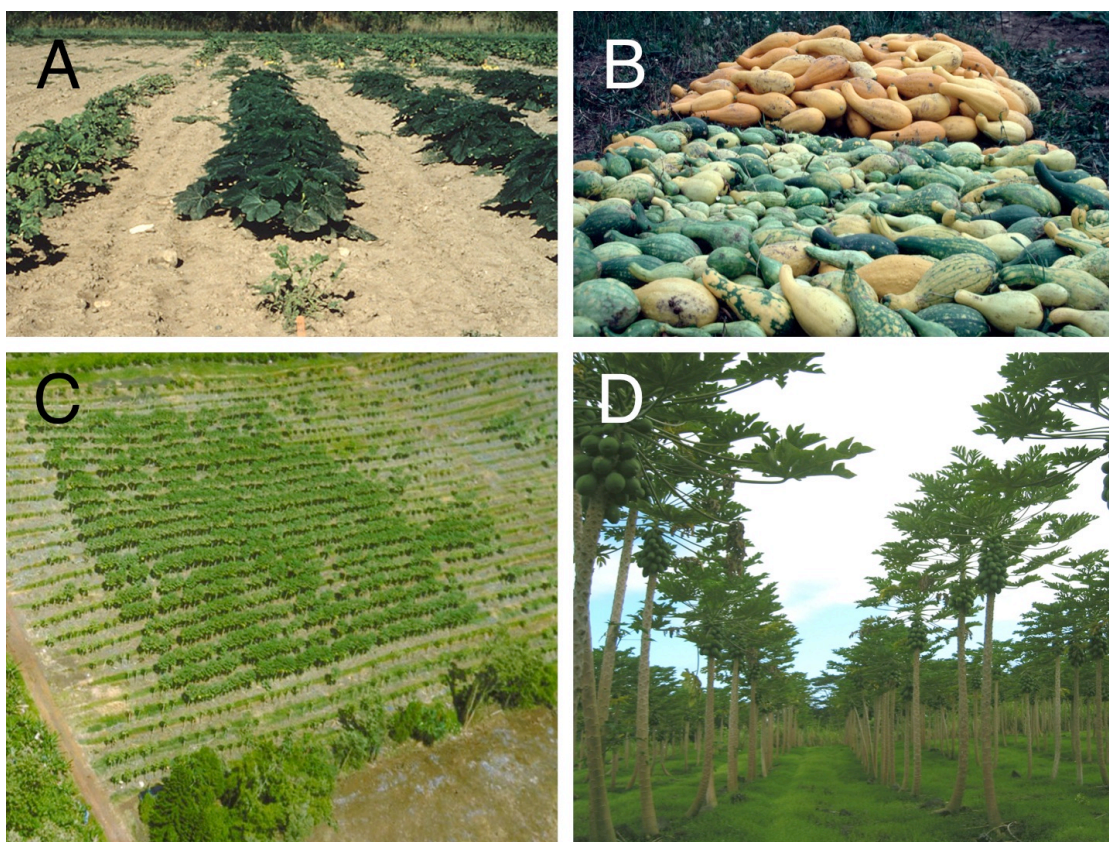
### **Virus-resistant summer squash**

Summer squash expressing the CP gene of ZYMV and WMV received exemption status in the USA in 1994 and was released thereafter. This was the first disease-resistant transgenic crop to be commercialized in the USA (**Table 5-1**). Plants of line ZW-20 are vigorous following exposure to aphid-mediated transmission of ZYMV and WMV (**Figure 5-1A**) and produce marketable fruits (**Figure 5-1B**) unlike conventional squash. Summer squash expressing the CP gene of CMV, WMV, and ZYMV was deregulated and commercialized in 1996. Subsequently, numerous squash types and cultivars have been developed by crosses and backcrosses with the two initially deregulated lines. Currently there are five zucchini and six straightneck or crookneck yellow squash cultivars for which combinations of resistance to ZYMV and WMV or resistance to CMV, ZYMV, and WMV are available.

The adoption of virus-resistant summer squash cultivars is steadily increasing since 1996. In 2006, the adoption rate was estimated to 22% (3,250 hectares) across the country with an average rate of 70% in New Jersey and 20% in Florida, Georgia, and South Carolina. The benefit to growers was estimated to \$24 million in 2006 (Johnson *et al.*, 2007).

**Table 5-1** Successful application of PDR in commercially available virus-resistant crops according to James (2009) and Stone (2008)

<b>Crop</b>	<b>Scientific name</b>	<b>Resistance to</b>	<b>Country of Release</b>
Papaya	<i>Carica papaya</i>	<i>Papaya ringspot virus</i>	USA, China
Pepper	<i>Capsicum</i>	<i>Cucumber mosaic virus</i>	China
Squash	<i>Cucurbita pepo</i>	<i>Cucumber mosaic virus</i>  <i>Watermelon mosaic virus</i>  <i>Zucchini yellow mosaic virus</i>	USA
Tomato	<i>Solanum lycopersicum</i>	<i>Cucumber mosaic virus</i>	China



**Figure 5-1** Reaction of summer squash and papaya to virus infection. (a) Resistance of transgenic summer squash ZW-20 (center and right rows) to aphid-mediated transmission of ZYMV and WMV from virus-infected conventional plants that served as inoculum source following mechanical inoculation (left row and first plant in the center row). (b) Comparative fruit yield of virus-resistant transgenic summer squash (back) and virus-infected conventional squash (front). (c) Aerial view of an experimental field of healthy transgenic PRSV-resistant Rainbow papaya (center) surrounded by rows of PRSV-infected conventional papaya (courtesy of D. Gonsalves). (d) Commercial field of PRSV-resistant papaya field in Hawaii.

### **Virus-resistant papaya**

Papaya expressing the coat protein gene of *Papaya ringspot virus* (PRSV) was deregulated in 1998 and commercialized in Hawaii (**Table 5-1**). PRSV is a major limiting factor to papaya production in Hawaii and around the world. After extensive experimental testing (**Figure 5-1C**), PRSV-resistant papaya was released in 1998 as devastation caused by the virus reached record proportions in the archipelago's main production region (Gonsalves, 1998). The impact of PRSV-resistant papaya on the papaya industry in Hawaii is evidenced by its rapid adoption rate (**Figure 5-1D**). In 2000, the first wave of transgenic papaya bore fruit on more than 42% of the total acreage (Johnson *et al.*, 2007). Resumption of fruitful harvests put papaya packing houses back in business and provided a \$4.3 million impact over a 6-year period (Fuchs, 2008).

By 2006, transgenic papaya cultivars were planted on more than 90% of the total papaya land in Hawaii (780 of 866 total hectares) (Johnson *et al.*, 2007), with the remaining conventional fruit shipped mainly to Japan, one of the major export countries for the Hawaiian papaya industry along with Canada (Suzuki *et al.*, 2007). After a decade of segregating transgenic and nontransgenic papaya fruits, this practice may be nearing end due to the recent deregulation of the transgenic fruit in Japan (D. Gonsalves, personal communication) following deregulation in Canada (Suzuki *et al.*, 2007).

### **Other examples**

Two virus-resistant potato lines were deregulated in 1998 and 2000 in the

USA. After failed attempts to create a potato line resistant to *Potato leafroll virus* (PLRV) by coat protein gene expression, lines expressing a PLRV replicase gene were created, field tested, deregulated, and commercialized (Kaniewski and Thomas, 2004). Later, this resistance was stacked with a synthetic *Cry* gene that conferred resistance to Colorado potato beetle. Another potato cultivar was developed by adding the coat protein gene of PVY. Although many growers in the Pacific Northwest, Midwest US and Canada were growing transgenic potato, and no resistance breakage was reported, nor any detrimental impact on the environmental or human health, virus-resistant potato were withdrawn from the market after the 2001 season due to the reluctance of several large processors and exporters to adopt these products (Kaniewski and Thomas, 2004).

In the People's Republic of China, tomato and sweet pepper resistant to CMV were released as well as papaya resistant to PRSV (James, 2009; Stone, 2008) (**Table 4-1**). Limited if any, information is available on their adoption rate.

Although not released yet, the plum cultivar 'Honeysweet' resistant to PPV is under consideration for deregulation in the USA. The US Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) has granted this cultivar deregulated status (Bech, 2007) and the Food and Drug Administration (FDA) has deemed a pre-market review of the 'Honeysweet' unnecessary. Presently, the Environmental Protection Agency (EPA) is examining deregulation petitions for 'Honeysweet.' Another PRSV-resistant papaya has been deregulated by two of the three US biotechnology regulatory authorities. Line X17-2 differs from the previously deregulated Hawaiian papaya in that it expresses the CP gene of a Florida isolate of



PRSV and is suitable for cultivation in Florida (Davis, 2004). APHIS and the FDA have granted X17-2 deregulated status (Anonymous, 2009; Shea, 2009). The realized economic benefits and minimal environmental hazards of the previously deregulated virus-resistant Hawaiian papaya figured prominently into APHIS' favorable consideration (Gregoire and Abel, 2008). The EPA will consider the plant pest risk of X17-2 after the developer submits a petition for deregulation.

### **Stability and durability of engineered virus resistance**

Plant viruses can evade the antiviral defense response by encoding RNA silencing-suppressor genes (Díaz-Pendón and Ding, 2008; Ding and Voinnet, 2007; Eamens *et al.*, 2008; Li and Ding, 2006; Voinnet, 2008). The HC-Pro protein of TEV and the 2b protein of CMV were amongst the first viral suppressors of transgene silencing identified (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998). Silencing suppressors from different plant viruses counteract various steps in the RNA silencing process (Díaz-Pendón and Ding, 2008; Ding and Voinnet, 2007; Li and Ding, 2006; Voinnet *et al.*, 1999). As a consequence, silencing-based resistance to one virus can be partially counteracted by infection with an unrelated virus carrying a silencing suppressor gene (Mitter *et al.*, 2003). Such an effect was not observed with plum trees expressing the PPV CP gene (Ravelonandro, 2007; Zagrai *et al.*, 2008) or summer squash expressing the CP genes of CMV, ZYMV, and WMV (Fuchs *et al.*, 1998; Tricoli *et al.*, 1995) following infection with heterologous viruses.

Transcriptional gene silencing and genetic background are two documented

variables that can cause transgenic plants expressing viral sequences to lack the expected resistance phenotype (Febres *et al.*, 2008). Wheat transformed with the coat protein gene or replicase gene of *Wheat streak mosaic virus* tended to display more severe symptoms and higher relative virus titers in the field compared to the nontransformed parent cultivar (Sharp *et al.*, 2002). These results suggested that environmental conditions can affect the stability of engineered resistance since all transgenic lines showed a recovery phenotype in greenhouse experiments. It is known that RNA silencing is inactive at low temperatures (Szittyá *et al.*, 2003). This provides good conceptual rationale to pyramid virus-derived transgenes with conventional resistance genes, which can be inactivated at high temperature (Wang *et al.*, 2009).

Resistance breakdown has not been reported in more than 10 years of commercial deployment of transgenic summer squash and papaya. Similarly, resistance is durable for PPV-resistant plum trees tested in experimental orchards over 13 years in Europe, despite constant exposure to viruliferous aphids vectoring diverse PPV populations (Capote *et al.*, 2007; Malinowski *et al.*, 2006; Ravelonandro, 2007; Zagari *et al.*, 2007). In contrast, resistance breakdown has been demonstrated in laboratory and greenhouse settings with papaya. Early work revealed that resistance to PRSV was narrow in cultivars expressing the CP gene from a Hawaiian isolate of PRSV; plants were resistant to PRSV isolates from Hawaii but largely susceptible to isolates outside of Hawaii, depending on the extent of sequence divergence (Suzuki *et al.*, 2007). Efforts to pyramid genes from highly conserved region of the PRSV genome from various isolates for broad-spectrum resistance are underway.

## ***DISCUSSION***

The concept of PDR was described a quarter century ago (Sanford and Johnston, 1985). This theory has provided a framework to engineer genetic constructs from a viral genome and use them as resistance genes to protect plants from virus infection. Application of this conceptual knowledge has introduced novel approaches for virus control by providing new means to develop resistant crop cultivars and increase opportunities to implement effective and sustainable management strategies of virus diseases. After its validation with TMV in tobacco plants in 1986, the concept of PDR has been applied successfully against a wide range of viruses in many plant species so that the past 25 years have witnessed an explosion in the development of virus-resistant transgenic plants.

Several virus-resistant transgenic crops resulting from the application of PDR have been extensively evaluated under field conditions and many more have been created and validated in laboratory or greenhouse conditions. The first resistant horticultural crops resulting from the application of PDR were vegetable (summer squash, sweet pepper, tomato, and potato) and fruit (papaya and plum) crops. Based on their efficacy at controlling virus diseases (Eamens *et al.*, 2008; Hily *et al.*, 2004; Prins *et al.*, 2008; Suzuki *et al.*, 2007; Tricoli *et al.*, 1995), a history of ready adoption by growers (Suzuki *et al.*, 2007) and no documented detrimental environmental impact (Fuchs and Gonsalves, 2007), more virus-resistant transgenic crops are likely to reach the market in the future. While several crop plants show good resistance to virus infection in the field, the dearth of commercialized examples beyond summer squash, papaya, tomato, and sweet pepper suggests that steep legal or regulatory issues, among

other issues, have barred market entry.

The dedication and perseverance on the part of a handful of researchers in the public and private sectors have extended PDR beyond an academic exercise to a proven technology for commercial use and efficient management of virus diseases. The creation and deployment of PRSV-resistant papaya have provided a safe and effective way to save an entire fruit industry on the Hawaiian Islands. The same could be true for Thailand but for negative intervention by an international nongovernmental organization (Davidson, 2008). Virus-resistant summer squash and potato have been deregulated in the USA but only summer squash remain commercially available to date. A virus-resistant plum and another virus-resistant papaya await full deregulation in the USA. The People's Republic of China is likewise moving forward with virus-resistant transgenic crops and has already commercialized virus-resistant sweet pepper, tomato, and papaya (James, 2009; Stone, 2008).

The application of the concept of PDR also paved the way for tremendous progress to be made at unraveling the biology of antiviral pathways of RNA silencing in plants, a natural and potent defense mechanism against viruses that can be triggered by the insertion and expression of viral gene constructs in susceptible hosts (Baulcombe, 2007; Eamens *et al.*, 2008; Lin *et al.*, 2007; Obbard *et al.*, 2009; Prins *et al.*, 2008; Voinnet, 2008). Knowledge of RNA silencing has provided new and unprecedented insights into virus–host interactions. dsRNA was identified as trigger of the antiviral defense mechanism, virus-encoded silencing suppressors as counterattack factors and symptom inducers, and pathogen-homing siRNAs as guides for the destruction of viral RNA by RISC (Baulcombe, 2007; Eamens *et al.*, 2008; Lin *et al.*,

2007; Obbard *et al.*, 2009; Voinnet, 2008). These developments stemming from the theory of PDR (Sanford and Johnston, 1985) shed light on the molecular and cellular mechanisms underlying engineered resistance in plants expressing virus-derived gene constructs.

The concept of PDR (Sanford and Johnston, 1985) provided unique opportunities for innovative solutions to control virus diseases by developing virus-resistant crops expressing genetic elements derived from a virus' own genome. A quarter century later, lessons from field experiments with various transgenic crops engineered for virus resistance and the commercial release of virus-resistant papaya, summer squash, sweet pepper, and tomato have conclusively demonstrated that applying the concept PDR is a practical strategy to mitigate the impact of virus diseases on agriculture.

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## CHAPTER 6

### ***GENOMIC BASIS OF BASAL VIRUS RESISTANCE***<sup>c</sup>

#### ***ABSTRACT***

The relationship between allopolyploidy and plant virus resistance is poorly understood. To determine the relationship of plant evolutionary history and basal virus resistance, a panel of *Nicotiana* species from diverse geographic regions and ploidy levels was assessed for resistance to non-coevolved viruses from the genus *Nepovirus*, family *Secoviridae*. The heritability of resistance was tested in a panel of synthetic allopolyploids. Leaves of different positions on each inoculated plant were tested for virus presence and a subset of plants was re-inoculated and assessed for systemic recovery. Depending on the host-virus combination, plants displayed immunity, susceptibility or intermediate levels of resistance. Synthetic allopolyploids showed an incompletely dominant resistance phenotype and manifested systemic recovery. Plant ploidy was weakly negatively correlated with virus resistance in *Nicotiana* species, but this trend did not hold when synthetic allopolyploids were taken into account. Furthermore, a relationship between resistance and geographical origin was observed. The gradients of resistance and virulence corresponded to a modified matching allele

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<sup>c</sup> A manuscript comprised of substantially similar content (Allopolyploidy and the evolution of plant virus resistance) by Gottula, J., Lewis, R., Saito, S. and Fuchs, M.) has been submitted for publication in *BMC Evolution* and is used here in accordance with this journal's copyright policy.

model of resistance. Intermediate resistance responses of allopolyploids corresponded with a model of multi-allelic additive resistance. The variable virus resistance of extant allopolyploids suggested that selection-based mechanisms surpass ploidy with respect to evolution of basal resistance to viruses.

## ***INTRODUCTION***

The ‘Red Queen Hypothesis’ suggests that coevolution between hosts and pathogens or pests results in a ‘boom and bust’ cycle where neither host nor its invader can gain lasting supremacy (Clay and Kover, 1996). Allopolyploidy could provide an opportunity for host species to outpace Red Queen coevolution and achieve epochal gains in resistance such as when two moderately-resistant diploids give rise to an allotetraploid with a full complement of resistance genes. This allopolyploid resistance hypothesis incorporates resistance into models explaining heterosis (Jackson and Tinsley, 2003; Prentis *et al.*, 2008), and has been tested experimentally in multiple plant and animal systems (Mouliia *et al.*, 1999; Wolinska *et al.*, 2008). Allopolyploidization contributes to 2-4% of speciation events in Angiosperms (Otto and Whitton, 2000).

Viruses have challenged plants for millennia (Mette *et al.*, 2002; Nawaz-ul-Rehman and Fauquet, 2009; Gibbs *et al.*, 2010). The genus *Nicotiana* has been used as a model system for studying plant-virus interactions and for investigating genotypic and phenotypic changes that occur at and after polyploidization (Lewis, 2011). The genus *Nicotiana* has 76 recognized species, 35 of which are allotetraploids arising from at least five independent interspecific allopolyploidization events (Lewis, 2011). The most likely diploid progenitors of most *Nicotiana* allopolyploids have been determined using nuclear and plastid DNA sequence information (Chase *et al.*, 2003; Kovarik *et al.*, 2004; Clarkson *et al.*, 2010; Kelly *et al.*, 2013). While the majority of *Nicotiana* allopolyploids retained their original chromosome number, most species in section *Suaveolentes* underwent a reduction in chromosome number. Genomic



changes can occur in the earliest generations following polyploidization (Patel *et al.*, 2011; Renny-Byfield *et al.*, 2011, Renny-Byfield *et al.*, 2012), and all well-studied *Nicotiana* allotetraploids have undergone gene loss or conversion (Kovarik *et al.*, 2004; Clarkson *et al.*, 2005). The main center of diversity for *Nicotiana* is Bolivia and the natural range of this genus extends throughout South America, to the Western US, Australia and Africa (Lewis, 2011). In particular, *N. tabacum* and *N. rustica* likely originated in South America, *N. clevelandii* and *N. quadrivalvis* are endemic to the Western US, and all but one species of section *Suaveolentes* are endemic to Australia (Lewis, 2011).

Plant viruses are commonly characterized by their experimental host ranges, sometimes incorporating reactions on *Nicotiana* species in their descriptions (Dawson and Hilf, 1992). The susceptibility status of *N. tabacum* is known for 541 plant viruses, and at least 29 *Nicotiana* species have been used in virus host range studies (Brunt *et al.*, 1996). Members of *Nicotiana* section *Suaveolentes* (such as *N. benthamiana*) tend to have the widest experimental host ranges. (Christie and Crawford, 1978; Dijk *et al.*, 1987; Dijk and Cuperus, 1989), and *N. benthamiana*'s multi-pathogen susceptibility makes it an important tool for phytopathology research (Goodin *et al.*, 2008; Lewis, 2011). Although the biological basis of *Nicotiana* nonhost resistance to viruses is unknown, a mutated form of *RNA-dependent RNA polymerase 1* in *N. benthamiana* compromises its broad-spectrum antiviral resistance response (Yang *et al.*, 2004). Several dominant, strain-specific virus resistance mechanisms have been described in *Nicotiana* (Holmes *et al.*, 1938; Cole *et al.*, 2001; Taliansky *et al.*, 1994), and closely related viruses exhibit differential capacities for

*Nicotiana* systemic infection (Cole *et al.*, 2001, Taliansky *et al.*, 1993)

Interspecific hybridization can be a useful tool for transferring resistance genes to crops species and for investigating virus resistance (Fraser, 1992; Dawson and Hilf, 1992; Maule *et al.*, 2007). Interspecific (euploid) hybrids of *Solanum tuberosum* and *S. brevidens* showed quantitative resistance to three diverse potato viruses compared to *S. tuberosum*, which exhibited high virus titers after inoculation (Pehu *et al.*, 1990). The broad-spectrum virus resistance was quantitatively enhanced if the hybrid contained additional copies of the *S. brevidens* genome or if the plants were aneuploids missing an *S. tuberosum* chromosome (Pehu *et al.*, 1990). Introgression of an alien chromosome from *N. africana* into *N. tabacum* produced tolerance (an amelioration of symptoms) to *Potato virus Y* in *N. tabacum*, but did not confer the immunity exhibited by *N. africana per se* (Lewis, 2005). These data support the conclusion that virus resistance is quantitatively controlled by multiple genes.

Nepoviruses are nematode-transmitted polyhedral-shaped viruses of the family *Secoviridae* (Sanfaçon *et al.*, 2009). These viruses, including *Grapevine fanleaf virus* (GFLV) and *Tomato ringspot virus* (ToRSV), have single-stranded, bipartite, RNA genomes in the positive-sense orientation. GFLV and ToRSV are present in most arable temperate regions and cause severe economic losses to grapevine and woody crops (Andret-Link *et al.*, 2004; Sanfaçon and Fuchs, 2011). Based on the distribution of their highly specific nematode vectors, the likely origins of GFLV and ToRSV are the Near East and Eastern North America, respectively (Raski *et al.*, 1983; Lamberti and Golden, 1984). *N. tabacum* exhibits a recovery reaction after infection of GFLV and ToRSV, and salicylic acid (SA)-based resistance mechanisms appear to be critical

for recovery from ToRSV (Dias and Harrison, 1963; Jovel *et al.*, 2011). RNA silencing mediates *N. tabacum* resistance (Ratcliff *et al.*, 1997; Siddiqui *et al.*, 2008) and tolerance (Yang *et al.*, 2004; Schwach *et al.*, 2005; Qu *et al.*, 2005; Ying *et al.*, 2010) to the nepoviruses *Tomato black ring virus* and *Tobacco ringspot virus*. Although RNA silencing- and SA-based mechanisms of nepovirus resistance have been described, no nepovirus resistance genes have been identified in *Vitis* spp. (Oliver *et al.*, 2011) or other plants, and the diversity and heritability of nepovirus resistance responses are unknown.

Although experimental work has shed light on the effect of allopolyploidy on pest resistance (Moullia *et al.*, 1999; Wolinska *et al.*, 2008), very little is currently known about how allopolyploidy could impact evolution of plant virus resistance. The objective of this research was to investigate the relationship between allopolyploidy, geographical origin and genomic bases of basal anti-nepoviral responses in *Nicotiana*. The *Nicotiana*-nepovirus pathosystem is a logical choice to test basal (nonspecific) antiviral responses because *Nicotiana* species are generally inbreeding (Lewis, 2011), nepovirus strains are genetically stable (Gottula *et al.*, 2013) and these plants and viruses have not coevolved. In this study, nepovirus resistance status of *Nicotiana* was tested and heritability of virus resistance using synthetic allopolyploids was ascertained. A local or systemically acquired resistance was also tested. The central hypothesis was that greater or lesser basal resistance could be explained by geography and ancestry, and that allopolyploids exhibit greater levels of virus resistance than diploids.

## **MATERIALS AND METHODS**

### **Plant material**

Seventeen *Nicotiana* species and seven synthetic allopolyploids (**Table 6-1**) were assayed for nepovirus resistance. With the exception of  $2x(N. \textit{tabacum} \times N. \textit{benthamiana})$ , an infertile amphihaploid, all genetic materials were self-fertile. The synthetic allopolyploids exhibited no obvious phenotypic segregation. Seeds of  $2x(N. \textit{tabacum} \times N. \textit{benthamiana})$  (DeVerna *et al.*, 1987) were a gift from Dr. G.B. Collins's research program (University of Kentucky, Lexington, KY). Seeds of *N. benthamiana*, *N. tabacum* cv. Xanthi and *N. clevelandii* were from Drs. D. Gonsalves and R. Provvidenti (Cornell University New York State Agricultural Experiment Station, Geneva, NY). Seeds of *N. rustica*, *N. glauca*, *N. glutinosa* and *N. sylvestris* were obtained from commercial sources. All of the other *Nicotiana* seeds were provided by the United States Nicotiana Germplasm Collection maintained at North Carolina State University (Raleigh, NC). Seedlings were grown in four-inch pots containing soilless potting media. Plants were grown in a greenhouse maintained at 24-26°C supplemented with high pressure sodium lamps for an 18 hour light/ 8 hour dark photoperiod, and watered daily or every other day as needed, and fertilized weekly.

### **Virus strains and inoculation procedure**

GFLV strain F13 from France (Ritzenthaler *et al.*, 1991; Serghini *et al.*, 1990) and strain GHu from Hungary (Huss *et al.*, 1989, Vigne *et al.*, 2013) were isolated from infected grapevines, and ToRSV strain AP was isolated from an infected apricot tree in New York State (Bitterlin and Gonsalves, 1988). GFLV and ToRSV strain AP

**Table 6-1** Sources of *Nicotiana* species and synthetic allopolyploids used in this study

<i>Nicotiana</i> sp.	Authority	Accession <sup>a</sup>	Germplasm source	PI #	Origin <sup>b</sup>	Provider <sup>c</sup>
<i>attenuata</i>	Torr. ex S. Watson	N/A	Bureau of Land Management	W6 27220	SW US Pinyon forest	NCSU
<i>goodspeedii</i>	H.-M. Wheeler	25-G	USDA ARS Beltsville	NSL 8663	Australia	NCSU
<i>obtusifolia</i>	Martens and Galeotti	TW9 8	USDA ARS Beltsville	555543	SW US/ NW Mex	NCSU
<i>debneyi</i>	Domin.	TW3 6	N/A	N/A	Australia	NCSU
<i>kawakamii</i>	Y. Ohashi	TW7 2	Iwata Experiment Station	459106	Bolivia	NCSU
<i>otophora</i>	Griseb.	TW9 7	Servicio Agricola Inter- Americano	302477	Ibanex Province, Bolivia	NCSU
<i>paniculata</i>	L.	TW1 00	C. Rick, Univ. California	241769	Peru	NCSU
<i>setchelii</i>	Goodsp.	TW1 21	USDA ARS Beltsville	555557	Peru	NCSU
<i>suaveolens</i>	Lehm.	TW1 28	CSIRO	230960	Australia	NCSU
<i>tomentosiformis</i>	Goodsp.	TW1 42	USDA ARS Beltsville	555572	Bolivia	NCSU
<i>glauca</i>	Graham	N/A	World Seed Supply, Mastic Beach, NY	N/A	Bolivia or Argentina	comm- ercial source
<i>glutinosa</i>	L.	N/A	World Seed Supply, Mastic Beach, NY	N/A	Bolivia, Ecuador, Peru	comm- ercial source
<i>rustica</i>	L.	N/A	World Seed Supply, Mastic Beach, NY	N/A	Bolivia, Ecuador or Peru	comm- ercial source
<i>sylvestris</i>	Speg. and Comes	N/A	Botanical Interests; Broomfield, CO	N/A	Bolivia or Argentina	comm- ercial source
<i>benthami- ana</i>	Domin.	N/A	N/A	N/A	Australia	Cornell
<i>clevelandii</i>	A. Gray	N/A	N/A	N/A	SW US	Cornell

<i>Nicotiana</i> sp.	Authority	Accession <sup>a</sup>	Germplasm source	PI #	Origin <sup>b</sup>	Provider <sup>c</sup>
<i>tabacum</i> cv. Xanthi	L.	N/A	N/A	N/A	Domestic- ated	Cornell
Hybrid maternal parent	Hybrid paternal parent	PI number	Hybrid accession reference	ploidy	Creator	Donor
<i>sylvestris</i>	<i>tomentos- iformis</i>	555722	TH37	amphidiploid	L. Burk; Prosser, WA	NCSU
<i>rustica</i> var. <i>brasilia</i>	<i>tabacum</i> cv. Boltons special	555701	TH34	amphidiploid	Anon.	NCSU
<i>sylvestris</i>	<i>otophora</i>	555721	TH32	amphidiploid	L. Burk; Prosser, WA	NCSU
<i>quadrival- vis</i>	<i>tabacum</i> cv. Red Russian	555515	TH1	amphidiploid	USDA ARS Beltsville	NCSU
<i>glutinosa</i>	<i>tabacum</i> cv. Red Russian	555520	TH10	amphidiploid	USDA ARS Beltsville	NCSU
<i>debneyi</i>	<i>clevelandii</i>	555699	TH15	amphidiploid	Cameron, UC Berkeley	NCSU
<i>tabacum</i> cv. Turkish SamS9-7	<i>bentham- iana</i>	N/A	hybrid 230	amphihaploid	G.B. Collins; Lexington , KY	KSU

<sup>a</sup> N/A: not available

<sup>b</sup> Specific origin of the accession is given, where known. Otherwise, the endemic range of the species according to Lewis (2011) is listed

<sup>c</sup> NCSU: North Carolina State University, KSU: Kentucky State University, Cornell: Cornell University New York State Agricultural Experiment Station  
were maintained in *N. benthamiana*. Virus inoculum was prepared by mechanically

inoculating *N. benthamiana* and storing infected tissue at -80°C until inoculation of the host panel. Infected *N. benthamiana* tissue was ground 1:10 (w:v) in inoculation buffer (15mM Na<sub>2</sub>HPO<sub>4</sub> and 35mM KH<sub>2</sub>PO<sub>4</sub> pH 7.0) using a steel grinding set in a tissue-lyser (Qiagen, Valencia, CA) and inoculated to three corundum-dusted leaves of each test plant with a ceramic pestle. Panels of four to 32 (median 17) plants per virus-host combination were selected for uniformity in size and mechanically inoculated when they had 4-5 leaves and were approximately 3 cm in height. All plants were rinsed with water five to ten minutes after inoculation.

### **Sampling and virus tests**

Plant tissue was collected from inoculated plants and processed for virus detection via double antibody-sandwich enzyme-linked immunosorbent assay (DAS-ELISA). Apical leaf positions were defined by counting nodes on the whorl upwards from the highest inoculated leaf. Apical leaves were collected at time points sufficient to detect cumulative virus infection: nine to 18 dpi for position one, 17 to 28 dpi for position two, and 26-60 dpi for position three. In plants where intermediate resistance phenotypes were observed, additional collections were made at 41 to 57 dpi for position four. Inoculated leaves were collected and processed between 21 and 54 dpi. Fresh tissue was ground in 1:10 (w:v) in 25mM sodium phosphate buffer using a semi-automated HOMEX 6 tissue homogenizer and mesh grinding bags (Bioreba, Reinach, Switzerland). DAS-ELISAs for GFLV and ToRSV were carried out in Nunc MaxiSorp<sup>®</sup> flat-bottom 96 well polystyrene microtiter plates (Fisher Scientific, Pittsburgh, PA) according to the manufacturer's protocol (Bioreba). Absorbance



(OD<sub>405nm</sub>) was measured after two hours of substrate incubation using a BioTek Synergy2 plate reader and Gen5 software was used to calculate blank-subtracted absorbance (Biotek, Winooski, VT). Each ELISA plate contained positive and negative checks, and the validity of each test was ascertained before data was processed. Samples were considered positive if their absorbance values were greater than two times the mean absorbance values of negative controls.

### **Evaluation of infection phenotypes**

Virus symptoms were monitored daily on inoculated and apical plant leaves. Leaf samples that were positive or negative in DAS-ELISA for GFLV or ToRSV in each inoculation group were counted and converted into percent infection at each leaf position. Six resistance categories were assigned based on the infection outcome in inoculated leaves and in successive apical leaves. Virus-host combinations that yielded no detectable virus in the inoculated leaf (and apical leaves) were designated as ‘immune’ (category 1). ‘Early recovery’ (category 2) was defined as any level of inoculated leaf infection (10% to 100%) but the virus was rarely or infrequently (<10%) detected in the first apical leaf. ‘Late recovery’ (category 3) was defined at 10% to 100% infection in the first or second apical leaf position but a decline in virus incidence at higher leaf axes. ‘Intermediate recovery’ (category 4) was defined as 20% to 80% infection frequencies in all leaf axes, and no clear pattern of reduction or expansion of virus incidence in successively higher axes. ‘Delayed susceptibility’ (category 5) was defined as a steady increase in virus incidence at successively higher apical leaf axes until the highest tested position contained >75% frequency of virus

infection. ‘Full susceptibility’ (category 6) was defined as virus incidence in 100% of apical leaves. The inoculated leaf was tested to discern immunity from early recovery.

### **Tests for systemic recovery**

A subset of the host panel exhibiting recovery from inoculation with GFLV strains F13 or GHu [4x(*N. sylvestris* x *N. tomentosiformis*), 4x(*N. glutinosa* x *N. tabacum*) and 4x(*N. sylvestris* x *N. otophora*)] was re-inoculated with GFLV-GHu in the fourth leaf position 34 days after the original inoculation. Re-inoculated leaves were tested for GFLV incidence at five dpi by DAS-ELISA. Negative values were interpreted as systemic recovery and positive values were interpreted as a lack of systemic recovery.

### **Statistics**

Statistics were computed on JMP version 10.0 (SAS Institute, Cary, NC). A score of one was assigned for each leaf infected in the first three apical leaf positions, and the sum of these scores are referred to as virus incidence. Each plant inoculated with a given virus was considered a replicate. Contingency analyses were used to compute Pearson’s correlations ( $r$ ) and contingency tables. Correlation analyses were made for species origin (South America, California, Australia or synthetic), ploidy ( $x=12$  to 48), and virus inoculum (GFLV-F13, GFLV-GHu or ToRSV-AP) with respect to virus incidence at each leaf position. Origin and virus inoculum was considered as categorical variables, ploidy as continuous and virus incidence as ordinal data. Correlation analyses were conducted where synthetic allopolyploids were either

included or excluded in the data set.

## **RESULTS**

### **Test for virus presence**

Twenty-four *Nicotiana* species and synthetic allopolyploids of distinct geographic origins were evaluated for their reaction to infection with GFLV strains GHu and F13, and ToRSV strain AP (**Table 6-1**). Since GFLV-GHu displays levels of virulence intermediate to that of GFLV-F13 and ToRSV-AP in most *Nicotiana* species, plants were primarily assessed for resistance to GFLV-GHu. Each plant-virus combination was sampled at three or more time points except when a definite resistance or susceptibility determination could be made in the first or second apical leaf i.e. for GFLV-F13-inoculated 4x(*N. sylvestris* x *N. tomentosiformis*), 4x(*N. glutinosa* x *N. tabacum*), 4x(*N. sylvestris* x *N. otophora*) and 4x(*N. rustica* x *N. tabacum*) (sampled once), and GFLV-GHu-inoculated 4x(*N. glutinosa* x *N. tabacum*) and *N. goodspeedii* (sampled twice). All panels were surveyed for virus presence in every plant [populations of four to 32 (median 17) plants], except for GFLV-F13-inoculated 2x(*N. tabacum* x *N. benthamiana*), where 23 plants in an original population of 70 plants was sampled for virus presence in apical leaves in a stratified sampling approach.

DAS-ELISA was used to determine virus presence or absence for 2719 GFLV samples and 536 ToRSV samples in 48 plant-virus combinations. DAS-ELISA reactions produced a bimodal distribution of absorbance values, which allowed a clear

delineation of virus-positive from virus-negative samples. Infection frequencies at each leaf position in each virus-host sample group were summed to calculate virus incidence, and this was used as the dependent variable in correlation analyses.

Correlations between sum virus incidence and two variables, ploidy and geographic origin, were computed for GFLV-GHu. Correlations between virus composition and sum virus incidence were also computed. Correlations were not made between ploidy or geographic origin and virus incidence levels for GFLV-F13 and ToRSV-AP inoculations, because limited inoculations were made with these viruses.

## Symptoms

Virus-inoculated plants were checked regularly for symptoms. The only instances of visible symptoms were for GFLV-GHu on *N. benthamiana*, *N. clevelandii*, *N. goodspeedii* and  $2x(N. tabacum \times N. benthamiana)$ , and for ToRSV-AP on *N. benthamiana* and  $2x(N. tabacum \times N. benthamiana)$ . GFLV-GHu symptoms on *N. benthamiana* and *N. clevelandii* were consistent with those previously described (Vigne *et al.*, 2013), and included vein clearing on *N. benthamiana* and amorphous ring-like mottling on *N. clevelandii*. GFLV-GHu symptoms on *N. goodspeedii* included vein clearing analogous to that observed for *N. benthamiana*. GFLV-GHu symptoms on the  $2x(N. tabacum \times N. benthamiana)$  amphihaploid were composed of non-necrotic ringspots on the first or second leaf position. ToRSV-AP symptoms on *N. benthamiana* were similar to those previously described (Jovel *et al.*, 2007), and included stunting, severe mottling, and necrosis from which the plant ultimately recovered. ToRSV-AP caused mild mottling and slight stunting on  $2x(N. tabacum \times N.$

*benthamiana*) but necrotic ringspots were not observed on *N. tabacum* cv. ‘Xanthi’.

### **Inoculated leaf infection**

DAS-ELISA revealed different frequencies of virus infection in inoculated leaves (**Figure 6-1**). Some host-virus combinations consistently produced absorbance values below the virus detection threshold, which reflects immunity or perhaps limited subliminal (single cell) infections. 4x(*N. sylvestris* x *N. tomentosiformis*), 4x(*N. sylvestris* x *N. otophora*), 4x(*N. glutinosa* x *N. tabacum*) and *N. paniculata* exhibited immunity to GFLV-F13 in inoculated leaves. Some host-virus combinations resulted in less than 50% inoculated leaf infection including GFLV-GHu-inoculated *N. obtusifolia* (13%) and *N. glauca* (14%), and GFLV-F13-inoculated 4x(*N. rustica* x *N. tabacum*) (43%) and 4x(*N. glutinosa* x *N. tabacum*) (44%) (**Table 6-2**). All other tested host-virus combinations produced 50% or greater inoculated leaf infection (**Table 6-2**). Since GFLV-GHu always produced infections in inoculated or apical leaves, and ToRSV-AP inoculations always produced some frequency of infection in the first apical leaf, there is no immunity within this *Nicotiana* panel to these two virus strains (**Table 6-3**).

### **High resistance interactions**

Virus-host combinations yielding no detectable virus in inoculated leaves (and apical leaves) were designated as immune (category 1). Immunity was observed for *N. paniculata*, 4x(*N. sylvestris* x *N. tomentosiformis*), 4x(*N. sylvestris* x *N. otophora*) and 4x(*N. glutinosa* x *N. tabacum*) inoculated with GFLV-F13 (**Figure 6-1**; **Table 6**

**Table 6-2** Plant responses to *Grapevine fanleaf virus* (GFLV) strains F13 and GHu, and *Tomato ringspot virus* (ToRSV) strain AP.

Table 6-2								
Virus	Sample size <sup>a</sup>	Species or synthetic allopolyploid	Resistance category <sup>b</sup>	Inoculated leaf <sup>c,d</sup>	Apical leaf 1	Apical leaf 2	Apical leaf 3	Apical leaf 4
GFLV-F13	25	4x( <i>N. sylvestris</i> x <i>N. tomentosiformis</i> )	1	0%	0%	N/T	N/T	N/T
	25	4x( <i>N. glutinosa</i> x <i>N. tabacum</i> )	1	0%	0%	N/T	N/T	N/T
	19	4x( <i>N. sylvestris</i> x <i>N. otophora</i> )	1	0%	0%	N/T	N/T	N/T
	6	<i>N. paniculata</i>	1	0%	0%	0%	0%	N/T
	10	<i>N. tabacum</i>	1 or 2	N/T	0%	0%	0%	N/T
	30	4x( <i>N. rustica</i> x <i>N. tabacum</i> )	2	43%	0%	N/T	N/T	N/T
	9	<i>N. otophora</i>	2	44%	0%	0%	0%	N/T
	6	<i>N. tomentosiformis</i>	2	67%	0%	0%	0%	N/T
	9	<i>N. debneyi</i>	2	89%	0%	0%	0%	N/T
	24	2x( <i>N. tabacum</i> x <i>N. benthamiana</i> )	3	69%	50%	4%	0%	N/T
	16	4x( <i>N. quadrivalvis</i> x <i>N. tabacum</i> )	3	100%	25%	19%	6%	N/T
	30	4x( <i>N. debneyi</i> x <i>N. clevelandii</i> )	4	N/T	70%	70%	67%	N/T
	24	<i>N. clevelandii</i>	6	100%	100%	100%	100%	N/T
	24	<i>N. benthamiana</i>	6	100%	100%	100%	100%	N/T
GFLV-GHu	14	<i>N. obtusifolia</i>	2	13%	0%	0%	0%	0%
	7	<i>N. glauca</i>	2	14%	0%	0%	0%	0%
	13	<i>N. sylvestris</i>	2	54%	0%	0%	8%	0%
	4	<i>N. kawakamii</i>	2	75%	0%	0%	0%	N/T
	23	<i>N. tabacum</i>	2	78%	0%	4%	0%	0%
	10	<i>N. tomentosiformis</i>	2	100%	0%	10%	10%	0%
	32	4x( <i>N. sylvestris</i> x <i>N. tomentosiformis</i> )	3	50%	16%	3%	3%	0%

Table 6-2								
Virus	Sample size <sup>a</sup>	Species or synthetic allopolyploid	Resistance category <sup>b</sup>	Inoculated leaf <sup>c,d</sup>	Apical leaf 1	Apical leaf 2	Apical leaf 3	Apical leaf 4
GFLV-GHu (continued)	30	4x( <i>N. quadrivalvis</i> x <i>N. tabacum</i> )	3	57%	10%	10%	3%	N/T
	10	<i>N. paniculata</i>	3	60%	10%	30%	0%	0%
	4	<i>N. setchellii</i>	3	75%	25%	0%	0%	N/T
	21	4x( <i>N. sylvestris</i> x <i>N. otophora</i> )	3	81%	10%	5%	0%	N/T
	8	<i>N. glutinosa</i>	3	100%	13%	0%	0%	N/T
	24	4x( <i>N. glutinosa</i> x <i>N. tabacum</i> )	3	100%	12%	4%	N/T	N/T
	30	2x( <i>N. tabacum</i> x <i>N. benthamiana</i> )	4	80%	50%	57%	60%	53%
	14	4x( <i>N. rustica</i> x <i>N. tabacum</i> )	4	100%	33%	24%	36%	N/T
	5	<i>N. rustica</i>	4	100%	80%	40%	60%	20%
	8	<i>N. debneyi</i>	4	100%	33%	89%	89%	88%
	7	<i>N. otophora</i>	5	100%	0%	56%	44%	78%
	24	<i>N. suaveolens</i>	5	N/T	4%	83%	79%	N/T
	19	4x( <i>N. debneyi</i> x <i>N. clevelandii</i> )	5	N/T	93%	93%	100%	N/T
	8	<i>N. attenuata</i>	6	100%	100%	100%	100%	N/T
	24	<i>N. clevelandii</i>	6	100%	100%	100%	100%	N/T
	24	<i>N. benthamiana</i>	6	100%	100%	100%	100%	N/T
	12	<i>N. goodspeedii</i>	6	N/T	100%	100%	N/T	N/T
ToRSV-AP	8	<i>N. otophora</i>	3	N/T	100%	0%	0%	N/T
	16	<i>N. tabacum</i>	3	N/T	88%	19%	6%	N/T
	16	<i>N. setchellii</i>	3	N/T	100%	13%	0%	N/T
	11	<i>N. kawakamii</i>	3	N/T	100%	27%	0%	N/T



Table 6-2								
Virus	Sample size <sup>a</sup>	Species or synthetic allopolyploid	Resistance category <sup>b</sup>	Inoculated leaf <sup>c,d</sup>	Apical leaf 1	Apical leaf 2	Apical leaf 3	Apical leaf 4
ToRSV-AP (continued)	12	4x( <i>N. rustica</i> x <i>N. tabacum</i> )	3	N/T	100%	100%	0	N/T
	23	4x( <i>N. sylvestris</i> x <i>N. tomentosiformis</i> )	3	N/T	100%	83%	0%	N/T
	27	4x( <i>N. sylvestris</i> x <i>N. otophora</i> )	3	N/T	100%	89%	26%	N/T
	16	2x( <i>N. tabacum</i> x <i>N. benthamiana</i> )	5	N/T	75%	81%	100%	N/T
	12	4x( <i>N. quadrivalvis</i> x <i>N. tabacum</i> )	6	N/T	100%	100%	100%	N/T
	24	<i>N. benthamiana</i>	6	N/T	100%	100%	100%	N/T

<sup>a</sup> The sample size denotes the lowest number of samples tested at any given time point to account for missing samples.

<sup>b</sup> Categories of resistance (1, most resistant, through 6, most susceptible) are indicated for each virus-host combination tested.

<sup>c</sup> Values represent the percent of plants in the sample showing detectable virus at each given leaf position.

<sup>d</sup>N/T: Not tested

**Table 6-3**      *Grapevine fanleaf virus* and *Tomato ringspot virus* resistance ratings of *Nicotiana* species and synthetic allopolyploids

Section <sup>a</sup>	Origin	x=	<i>Nicotiana</i> species	GFLV- GHu <sup>b</sup>	GFLV- F13 <sup>c,d</sup>	ToRSV- AP
<i>Tomentosae</i>	Bolivia	12	<i>N. otophora</i>	5	2	3
	Bolivia	12	<i>N. tomentosiformis</i>	2	2	
	Bolivia	12	<i>N. kawakamii</i>	2		3
	Peru	12	<i>N. setchellii</i>	3		3
<i>Paniculatae</i>	Peru	12	<i>N. paniculata</i>	3	1	
<i>Noctiflorae</i>	Bolivia	12	<i>N. glauca</i>	2		
<i>Sylvestres</i>	Bolivia	12	<i>N. sylvestris</i>	2		
<i>Undulatae</i>	Peru	12	<i>N. glutinosa</i>	3		
<i>Trigonophyllae</i>	SW US	12	<i>N. obtusifolia</i>	2		
<i>Petunioides</i>	SW US	12	<i>N. attenuata</i>	6		
<i>Suaveolentes</i>	Australia	16	<i>N. suaveolens</i>	5		
	Australia	19	<i>N. benthamiana</i>	6	6	6
	Australia	20	<i>N. goodspeedii</i>	6		
	Australia	24	<i>N. debneyi</i>	4	2	
<i>Polydicliae</i>	SW US	24	<i>N. clevelandii</i>	6	6	
<i>Rusticae</i>	Bolivia	24	<i>N. rustica</i>	4		
<i>Nicotiana</i>	Domesticated	24	<i>N. tabacum</i>	2	1-2	3
Wide crosses (synthetic allopolyploids)		24	4x( <i>N. sylvestris</i> x <i>N. otophora</i> )	3	1	3
		24	4x( <i>N. sylvestris</i> x <i>N. tomentosiformis</i> )	3	1	3
		36	4x( <i>N. glutinosa</i> x <i>N. tabacum</i> )	3	1	

Section <sup>a</sup>	Origin	x=	<i>Nicotiana</i> species	GFLV- GHu <sup>b</sup>	GFLV- F13 <sup>c,d</sup>	ToRSV- AP
Wide crosses (synthetic allopolyploids) continued		43	2x( <i>N. tabacum</i> x <i>N. benthamiana</i> )	4	3	5
		48	4x( <i>N. quadrivalvis</i> x <i>N. tabacum</i> )	3	3	6
		48	4x( <i>N. rustica</i> x <i>N. tabacum</i> )	4	2	3
		48	4x( <i>N. debneyi</i> x <i>N. clevelandii</i> )	5	4	

<sup>a</sup> Species and synthetic allopolyploids are referenced by their sections within the genus *Nicotiana*, primary location of origin, and their haploid chromosome numbers according to Lewis (2011)

<sup>b</sup> Categories of resistance (1, most resistant, through 6, most susceptible) are indicated for each virus-host combination tested.

<sup>c</sup> Boxes without resistance ratings represent virus-host combination not tested

<sup>d</sup> Category '1-2' denotes category 1 or 2 resistance could be operational

3). GFLV-F13-inoculated *N. tabacum* did not produce apical leaf infection, but whether this plant is immune (category 1) or possesses early recovery (category 2) to GFLV-F13 is uncertain because inoculated leaves were not tested. All tested members of section *Tomentosae*, *N. debneyi* and 4x(*N. rustica* x *N. tabacum*) exhibited early recovery (category 2) to GFLV-F13 (**Table 6-3**). *N. obtusifolia*, *N. glauca*, *N. sylvestris*, *N. kawakamii*, *N. tabacum* and *N. tomentosiformis* exhibited early recovery after GFLV-GHu inoculation. Early recovery was not observed for these species in response to inoculation with ToRSV-AP (**Table 6-3**).

### **Moderate resistance interactions**

Late recovery (category 3) was the most frequent host-virus interaction phenomenon observed in this test panel, and was seen for all virus isolates tested. All tested members of section *Tomentosae*, 4x(*N. rustica* x *N. tabacum*), *N. tabacum* and resynthesized allopolyploids involving possible *N. tabacum* progenitor species [4x(*N. sylvestris* x *N. tomentosiformis*) and 4x(*N. sylvestris* x *N. otophora*)] showed late recovery to ToRSV-AP (**Table 6-3**). 2x(*N. tabacum* x *N. benthamiana*) and 4x(*N. quadrivalvis* x *N. tabacum*) showed late recovery to GFLV-F13, and 4x(*N. quadrivalvis* x *N. tabacum*), 4x(*N. sylvestris* x *N. otophora*), 4x(*N. sylvestris* x *N. tomentosiformis*), 4x(*N. glutinosa* x *N. tabacum*), *N. glutinosa*, *N. paniculata* and *N. setchellii* showed late recovery to GFLV-GHu (**Table 6-3**). Intermediate recovery (category 4), characterized by fluctuation of virus incidence over three or more leaf axes (typically between 33% and 67%, **Table 6-2**), was observed in GFLV-GHu-inoculated *N. debneyi*, *N. rustica*, 4x(*N. rustica* x *N. tabacum*) and 2x(*N. tabacum* x *N.*

*benthamiana*), and in GFLV-F13-inoculated 4x(*N. debneyi* x *N. clevelandii*) (**Table 6-3**).

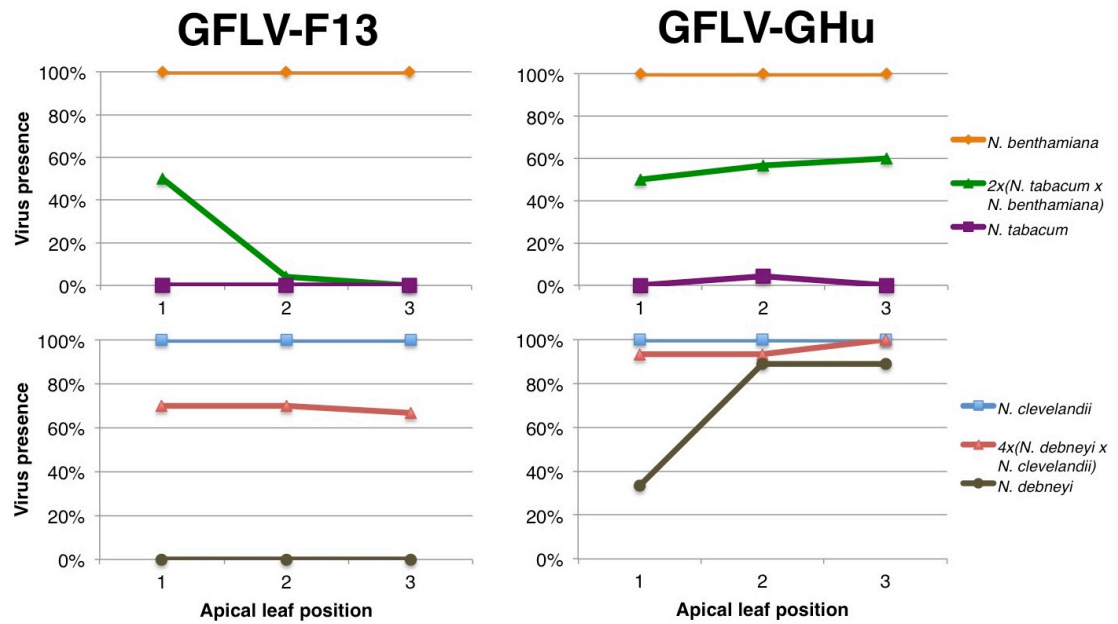
#### **Low or no resistance interactions**

Delayed susceptibility (category 5) was observed only in response to GFLV-GHu inoculation of *N. otophora*, *N. suaveolens*, and 4x(*N. debneyi* x *N. clevelandii*) (**Figure 6-1; Table 6-3**). Plants were designated as susceptible (category 6) when 100% of the plants became infected and virus was present in all tested leaves. *N. benthamiana* and *N. clevelandii* were susceptible to GFLV-F13 and GFLV-GHu, as expected (Vigne *et al.*, 2013), *N. goodspeedii* and *N. attenuata* were susceptible to GFLV-GHu, and *N. benthamiana* and 4x(*N. quadrivalvis* x *N. tabacum*) were susceptible to ToRSV-AP (**Figure 6-1; Table 6-3**).

#### **Additive resistance phenomena in synthetic polyploid plants**

Incompletely dominant virus resistance was observed in synthetic *Nicotiana* allopolyploids. Whereas *N. tabacum* showed high resistance to GFLV-GHu, ToRSV-AP and GFLV-F13, and *N. benthamiana* was fully susceptible to all three virus strains, 2x(*N. tabacum* x *N. benthamiana*) exhibited delayed susceptibility to GFLV-GHu, intermediate recovery to ToRSV-AP, and late recovery to GFLV-F13 (**Figure 6-2; Table 6-3**). *N. debneyi* exhibited early recovery to GFLV-F13 and intermediate recovery to GFLV-GHu. These differential resistance responses also appeared in 4x(*N. debneyi* x *N. clevelandii*), which exhibited intermediate recovery to GFLV-F13 and





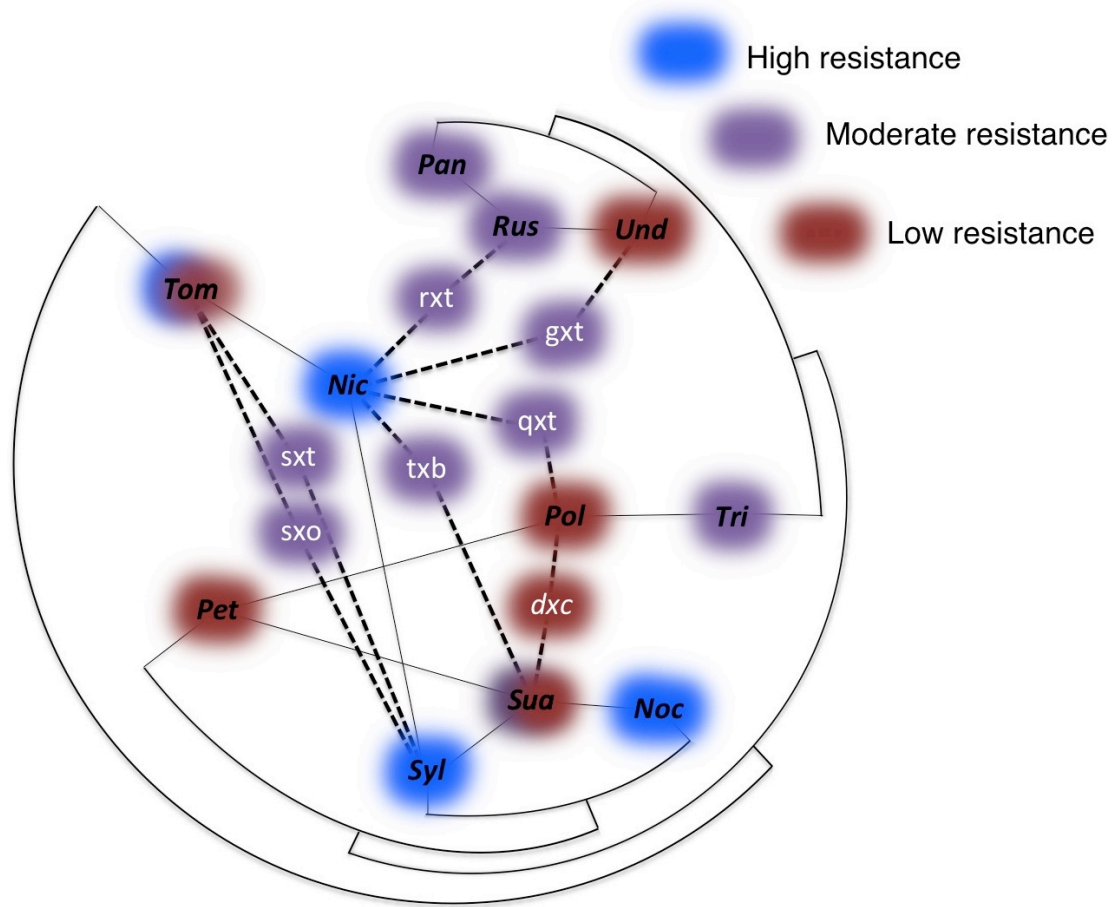
**Figure 6-2** Effect of synthetic *Nicotiana* allopolyploids on resistance to *Grapevine fanleaf virus* (GFLV) strains F13 (left panels) and GHu (right panels). *N. tabacum*, *N. benthamiana* and the  $2x(N. tabacum \times N. benthamiana)$  amphihaploid (upper panels); and *N. debneyi*, *N. clevelandii* and  $4x(N. debneyi \times N. clevelandii)$  allopolyploid (lower panels) were tested for additive resistance.



delayed susceptibility to GFLV-GHu (**Figure 6-2, Table 6-3**). Whereas *N. clevelandii* was fully susceptible to all viruses tested, and *N. debneyi* exhibited early recovery to GFLV-F13 and intermediate recovery to GFLV-GHu,  $4x(N. debneyi \times N. clevelandii)$  exhibited intermediate recovery to GFLV-F13 and delayed susceptibility to GFLV-GHu (**Figure 6-2, Table 6-3**). The  $4x(N. rustica \times N. tabacum)$  response to GFLV-GHu was not categorically different than the response of *N. rustica* (both category 4), but the synthetic allopolyploid showed consistently lower incidence of infection in apical leaves (23-40%) compared to *N. rustica* (40-80%), which could reflect the contribution of *N. tabacum* (category 2) to resistance (**Figure 6-1; Table 6-3**). The intermediate virus resistance observed across *Nicotiana* lineages (**Figure 6-3**) suggests quantitative resistance is not due to a single gene with dosage effects, but due to multiple genes with dosage effects.

### **Resistance profiles of allopolyploids and their progenitors**

Two natural allopolyploids (*N. clevelandii* and *N. tabacum*) and the closest relatives of their known progenitors were tested for GFLV-GHu resistance. The closest extant diploid progenitors of *N. clevelandii* are *N. obtusifolia* (maternal genome donor) and *N. attenuata* (paternal genome donor) (Clarkson *et al.*, 2010). While *N. obtusifolia* exhibited an early recovery phenotype (category 2), both *N. clevelandii* and *N. attenuata* showed complete susceptibility (**Figure 6-1; Table 6-3**). *N. tabacum*, its representative maternal genome donor (*N. sylvestris*), and one possible representative paternal genome donor (*N. tomentosiformis*) each exhibited early recovery (category 2). *N. otophora*, another representative of the possible *N. tabacum*



**Figure 6-3** *Grapevine fanleaf virus* strain GHu (GFLV-GHu) resistance categories superimposed on a *Nicotiana* phylogenetic tree modified from Clarkson *et al.* (2010) (curved lines), containing sections (abbreviated in black lettering) with allopolyploid ancestries as established by Clarkson *et al.* (2010) and Kelly *et al.* (2013) (solid straight lines). Shading surrounding sections denote the resistance category of representative species tested for GFLV-GHu resistance: blue (category 2, early recovery), purple (categories 3 and 4, late or intermediate recovery), or red (categories 5 and 6, delayed or full susceptibility). Representative *Nicotiana* species (sections) used in this study are *N. paniculata* (Paniculatae, 'Pan'), *N. rustica* (Rusticae, 'Rus'), *N. obtusifolia* (Trigonophyllae, 'Tri'), *N. benthamiana*, *N. debneyi*, *N. suaveolens* and *N. goodspeedii* (Suaveolentes, 'Sua'), *N. clevelandii* (Polydicliae, 'Pol'), *N. glauca* (Noctiflorae, 'Noc'), *N. sylvestris* (Sylvestres 'Syl'), *N. tabacum* (Nicotiana 'Nic'), *N. glutinosa* (Undulatae, 'Und') *N. attenuata* (Petunioides, 'Pet'), (Tomentosae, 'Tom')

including *N. kawakamii*, *N. otophora*, *N. setchellii* and *N. tomentosiformis*. Members of *Tomentosae* and *Suaveolentes* exhibited different GFLV-GHu resistance profiles and are accordingly dually or triply colored. Descent of synthetic allopolyploids used in this study (white letters) is indicated by dashed lines: 4x(*N. sylvestris* x *N. tomentosiformis*) ('sxt'), 4x(*N. rustica* x *N. tabacum*) ('rxt'), 4x(*N. glutinosa* x *N. tabacum*) ('gxt'), 2x(*N. tabacum* x *N. benthamiana*) ('txb'), 4x(*N. quadrivalvis* x *N. tabacum*) ('qxt') and 4x(*N. debneyi* x *N. clevelandii*) ('dxc').

paternal genome donors exhibited delayed susceptibility (category 5) to GFLV-GHu. Resynthesized allopolyploids corresponding to either *N. tabacum* ancestry scenario exhibited late recovery phenotypes (category 3) GFLV-GHu, with low virus incidence levels (**Figure 6-1; Table 6-2**). Thus, *N. tabacum* exhibits an early recovery phenotype similar to that of its maternal genome donor and of *N. tomentosiformis*, but less than that of *N. otophora* or representative resynthesized allopolyploids. Additionally, species of section *Suaveolentes* showed low or occasionally moderate resistance to GFLV-GHu, while its most closely related proposed paternal genome donor (*N. sylvestris*) (Kelly *et al.*, 2013) showed high resistance (early recovery) (**Table 6-3**). While neoallopolyploids showed intermediate GFLV-GHu resistance characteristics, extant allopolyploids did not show intermediate GFLV-GHu resistance characteristics (**Figure 6-3**).

### Systemic recovery

Systemic recovery was tested in apical leaves of GFLV-resistant (categories 1 or 3) synthetic allopolyploids  $4x(N. sylvestris \times N. tomentosiformis)$ ,  $4x(N. glutinosa \times N. tabacum)$ , and  $4x(N. sylvestris \times N. otophora)$  (**Table 6-4**). Resistance was induced with GFLV-GHu or GFLV-F13, and one upper, apical leaf of each recovered plant was re-inoculated with GFLV-GHu and tested for virus presence. Notably, plants that showed inoculated leaf susceptibility to GFLV-GHu lost this susceptibility in the apical leaf of the recovered plant, no matter whether the resistance was induced with GFLV-F13 or GFLV-GHu (**Table 6-4**). GFLV-GHu was occasionally detected in the apical inoculated leaf of GFLV-GHu-recovered plants encompassing two of 21 plants

**Table 6-4** Systemic recovery from *Grapevine fanleaf virus* (GFLV) strains F13 and GHu.

	1st inoculation <sup>a</sup>	Resistance response	Systemic recovery <sup>b</sup>
4x( <i>N. sylvestris</i> x <i>N. tomentosiformis</i> )	GFLV-GHu	Late recovery	90%
	GFLV-F13	Immunity	100%
4x( <i>N. glutinosa</i> x <i>N. tabacum</i> )	GFLV-GHu	Late Recovery	100%
	GFLV-F13	Immunity	100%
4x( <i>N. sylvestris</i> x <i>N. otophora</i> )	GFLV-GHu	Late Recovery	89%

<sup>a</sup> GFLV-GHu was inoculated to the fourth apical leaf following induction of resistance (resistance response against the virus in the 1st inoculation is indicated)

<sup>b</sup> Plants (n = 9 to 29) were characterized as having systemic recovery if GFLV was undetectable at five days post-inoculation.

in 4x(*N. sylvestris* x *N. tomentosiformis*) and one of nine plants in 4x(*N. sylvestris* x *N. otophora*). Of the plants that did not acquire systemic recovery, we cannot exclude the possibility of the originally-inoculated GFLV-GHu infecting these apical leaves, given late recovery does not bar the virus from infecting the fourth leaf position, albeit at a low incidence.

### **Relationship between host geographic origin and virus resistance**

Australian and North American accessions generally displayed greater levels of susceptibility than South American accessions to all virus strains tested (**Table 6-3**). The Australian species *N. benthamiana* and the North American species *N. clevelandii* were fully susceptible to all viruses tested, and Australian species *N. debneyi*, *N. suaveolens* and *N. goodspeedii*, and North American species *N. attenuata* and *N. quadrivalvis* displayed lower levels of resistance than South American species to GFLV-GHu individually or in hybrid backgrounds (**Table 6-3**). Exceptions to these geography-based resistance trends included the *N. debneyi* (Australia) early recovery response to GFLV-F13, the *N. obtusifolia* (North America) early recovery response to GFLV-GHu, and the *N. otophora* (South America) delayed susceptibility response to GFLV-GHu. Overall, origin had a significant ( $P<0.0001$ ) and moderate correlation for GFLV-GHu virus incidence when hybrids were excluded from the analysis ( $r=0.683$ ) and a weaker correlation ( $r=0.5422$ ,  $P<0.0001$ ) when hybrids were included, with South American species showing greater resistance than Australian species, which showed greater resistance than species from the Southwest US.

### **Limited relationship between host ploidy level and virus resistance**

There was a weak association between ploidy level and virus susceptibility. For example,  $n=12$  diploids from section *Tomentosae* generally displayed greater levels of resistance than  $n=16-24$  allopolyploids of section *Suaveolentes*, and similar levels of resistance to *N. tabacum* and *N. rustica* ( $n=24$ ) (**Table 6-3**). The correlation between GFLV-GHu virus incidence and chromosome number was low ( $r=-0.036$ ) and nonsignificant ( $P=0.2597$ ) when hybrids were included in the analysis, and low ( $r=-0.286$ ) but significant ( $P<0.0001$ ) when hybrids were excluded, indicating that increasing ploidy is weakly negatively related to GFLV-GHu virus incidence among extant *Nicotiana* species. These results indicate that increasing ploidy is correlated with slightly greater virus susceptibility, but that the trend is abolished when synthetic allopolyploids are taken into account.

### **Other trends in virus resistance**

Members of section *Tomentosae* produced higher inoculated leaf infection rates (75-100%) for GFLV-GHu than for GFLV-F13 (44-67%) (**Table 6-2**). Every tested member of section *Tomentosae* produced an early recovery phenotype for GFLV-F13 and a late recovery phenotype for ToRSV-AP (**Table 6-3**). Members of section *Tomentosae* showed variability in response to GFLV-GHu, where *N. kawakamii* and *N. tomentosiformis* exhibited early recovery, *N. setchellii* displayed late recovery, and *N. otophora* showed delayed susceptibility (**Table 6-3**). The delayed susceptibility of *N. otophora* to GFLV-GHu was masked in the  $4x(N. sylvestris \times N. otophora)$  synthetic allopolyploid, which reflected the early recovery of *N. sylvestris*

to GFLV-GHu (**Table 6-3**). Early recovery was also observed for *N. tabacum* inoculated with GFLV-GHu, a species believed to have evolved from a *N. sylvestris* x *N. otophora* or *N. sylvestris* x *N. tomentosiformis* hybridization event (Lewis, 2011). Members of section *Suaveolentes* exhibited intermediate or low resistance to the nepovirus strains tested, except for *N. debneyi*, which displayed early recovery after inoculation with GFLV-F13 (category 2) (**Table 6-3**).

*N. tabacum* and its corresponding resynthesized allopolyploids [ $4x(N. sylvestris$  x *N. otophora*) and  $4x(N. sylvestris$  x *N. tomentosiformis*)] exhibited high or moderate virus resistance phenotypes for each virus tested (**Table 6-3**). Both resynthesized allopolyploids are immune to GFLV-F13, and *N. tabacum* also displays high resistance to this virus. *N. tabacum* and its resynthesized allopolyploids showed late recovery to ToRSV-AP, though *N. tabacum* frequently had lower frequencies of infection at any given leaf position than its corresponding neoallopolyploids (**Figure 6-1**; **Table 6-2**). The response of *N. tabacum* and the synthetic allopolyploids  $4x(N. sylvestris$  x *N. otophora*) and  $4x(N. sylvestris$  x *N. tomentosiformis*) to GFLV-GHu were similar in terms of inoculated leaf infection, but *N. tabacum* showed early recovery whereas the neoallopolyploids showed late recovery, though the overall apical virus incidence levels were similar (**Table 6-2 and 6-3**). The recovery responses of *N. tabacum* to GFLV and ToRSV inoculation confirm previous reports (Dias and Harrison, 1963; Jovel *et al.*, 2011).

Synthetic polyploids formed from resistant and susceptible species frequently displayed resistance in the moderate categories (**Figure 6-3**).  $2x(N. tabacum$  x *N. benthamiana*) and  $4x(N. debneyi$  x *N. clevelandii*) exhibited intermediate resistance



phenotypes after inoculation with GFLV-GHu and GFLV-F13 as compared to their parents (**Figure 6-2**; **Table 6-3**). The same was true for the  $2x(N. benthamiana \times N. tabacum)$  response to ToRSV-AP (**Table 6-3**). An intermediate level of apical leaf infection was also seen in the  $4x(N. rustica \times N. tabacum)$  response to GFLV-GHu (**Figure 6-1**). ToRSV-AP typically produced equal or greater categorical ratings than GFLV-GHu, and GFLV-GHu always produced equal or higher category ratings than GFLV-F13 (**Table 6-3**). An exception to this virulence trend was that *N. otophora* and  $4x(N. rustica \times N. tabacum)$  showed lower resistance (higher category ratings) to GFLV-GHu than to ToRSV-AP (**Table 6-3**). Virulence differences between GFLV-F13 and GFLV-GHu were highly apparent in synthetic allopolyploid plants with resistant and susceptible parents, including  $2x(N. tabacum \times N. benthamiana)$ ,  $4x(N. rustica \times N. tabacum)$ , and  $4x(N. debneyi \times N. clevelandii)$  (**Table 6-3**; **Figure 6-2**). There was a significant ( $P < 0.0001$ ) but weak ( $r = 0.406$ ) correlation between virus composition and infection frequencies across plant genotypes (species or synthetic allopolyploids).

According to individual components of  $\chi^2$  in the contingency table that compared observed and expected virus incidence frequencies for each virus at each leaf position, there is a higher virus incidence in the first apical leaf than expected for ToRSV-AP; conversely, there is less virus incidence in the first apical leaf than expected for GFLV-F13 (data not shown). Expected and observed apical virus incidence values are similar for GFLV-GHu. These results suggest that ToRSV-AP displays higher virulence and GFLV-F13 displays lower virulence than GFLV-GHu in this panel of *Nicotiana* species.

## **DISCUSSION**

A spectrum of plant resistance and viral virulence was observed in the *Nicotiana-nepovirus* present panel. While all host-virus combinations exhibiting low leaf inoculation frequencies (<50%) exhibited early recovery, this phenotype was frequently associated with a high infection frequency (>50%) in inoculated leaves (**Table 6-2**). Moderate or high leaf inoculation frequencies ( $\geq 50\%$ ) were associated with an entire range of resistance and susceptibility phenotypes (category 2 through category 6) (**Figure 6-1**). Within individual plant genotypes, ToRSV-AP generally produced higher susceptibility ratings than GFLV-GHu, and GFLV-GHu always produced an equal or greater susceptibility rating than GFLV-F13 (**Table 6-3**), and the correlation between virus identity and virus incidence ratings were significant. The spectra of quantitative resistance displayed by *Nicotiana* accessions and virulence among nepoviruses suggest the role of multiple interacting alleles from *Nicotiana* accessions and nepoviruses in the determination of the ultimate infection outcomes. Similar plant genotype by virus genotype interactions were observed in a panel of 21 *Arabidopsis* accessions challenged with three *Cucumber mosaic virus* isolates (Pagán *et al.*, 2007).

The full susceptibility seen for 4x(*N. quadrivalvis* x *N. tabacum*) and delayed susceptibility of the 2x(*N. tabacum* x *N. benthamiana*) responses to ToRSV raises the interesting possibility that *N. quadrivalvis* and *N. benthamiana* may possess a dominant ToRSV susceptibility factor in *N. tabacum* backgrounds. The observation of ringspot symptoms on the GFLV-GHu-inoculated 2x(*N. tabacum* x *N. benthamiana*) amphihaploid suggests that the vein clearing symptomology typical of *N. benthamiana*

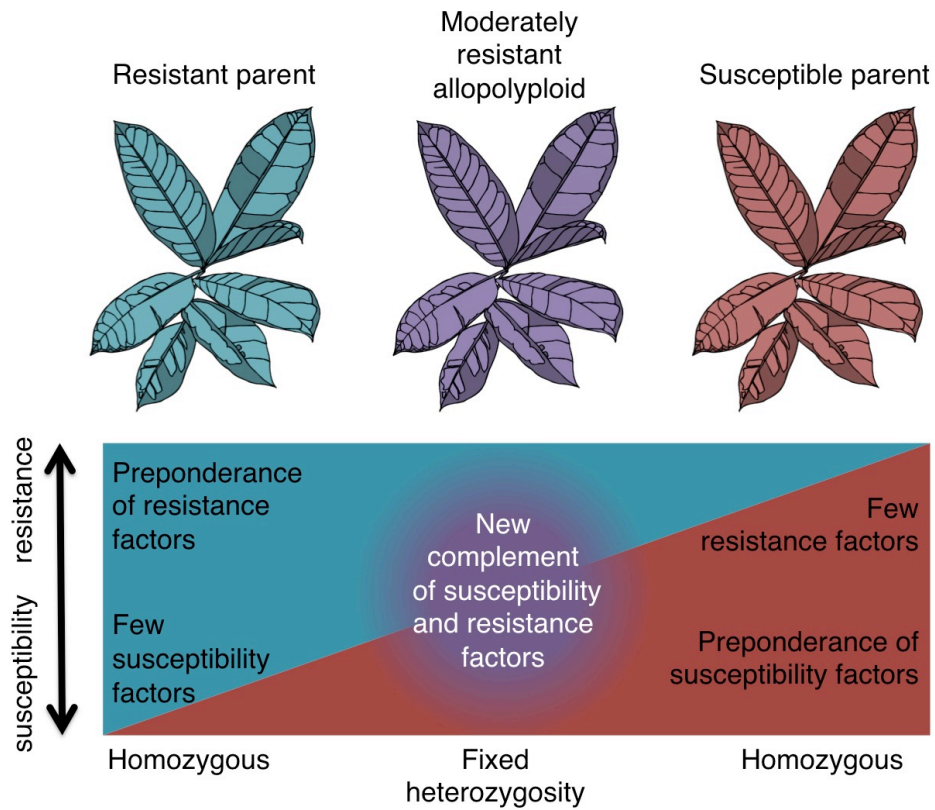
infection (Vigne *et al.*, 2013) is a recessive trait. Similarly, while ToRSV-AP produced necrosis on *N. benthamiana*, necrosis was not observed on the 2x(*N. tabacum* x *N. benthamiana*) amphihaploid or on *N. tabacum*. The absence of *N. tabacum*-ToRSV necrotic ringspot symptoms was unexpected given previous reports (Ross, 1961; Jovel *et al.*, 2011). The lack of hypersensitive responses observed in this host panel is consistent with the lack of involvement of a specific gene-for-gene recognition system in *Nicotiana*-GFLV and *Nicotiana*-ToRSV interactions. This lack of hypersensitive response and the absence of coevolutionary history between *Nicotiana* and GFLV or ToRSV supports the idea that resistance or susceptibility is due to the interaction of broad-spectrum immune responses and virulence factors (Schulze-Lefert and Panstruga, 2011).

Most plants in the host panel used in this study recovered from virus infection after infection was initially established in inoculated leaves. Recovery from virus infection is common, can be controlled by simple or complex host plant genetics, and can be countered by effective pathogen virulence factors (Gunduz *et al.*, 2004; Bruening, 2006; Maule *et al.*, 2007). Host plant and pathogen genotype determined the level of plant recovery to GFLV (**Figure 6-2**). Compatibility between host and viral components is a prerequisite for infection in the matching allele model (Lambrechts *et al.*, 2006; Fraile and García-Arenal, 2010). The partial resistance phenotypes observed in this study do not fit with the strict bimodality of the matching allele concept. However, a modified matching allele model that allows for partial compatibility and limited infection (**Figure 6-4**) (Clay and Kover, 1996; Fraile and García-Arenal, 2010) could explain the range of resistance and virulence observed in the *Nicotiana*-

nepovirus interactions observed here.

The intermediate resistance responses of *Nicotiana* neoallopolyploids are congruent with the additive resistance hypothesis proposed by Fritz *et al* (1999). By applying the modified matching allele model to the additive resistance hypothesis, we theorize that susceptible parents contribute susceptibility alleles and resistant parents contribute resistance alleles, and their neoallopolyploids contain novel combinations of resistance and susceptibility factors (**Figure 6-4**). Neoallopolyploids would possess a greater number of matching alleles than their more resistant parent, and the dosage of resistance factors would be reduced compared to the resistant parent. Furthermore, non-additive gene expression, which is commonly observed in allopolyploids and other hybrids (Chen, 2007; Rapp *et al.*, 2009; Scascitelli *et al.*, 2010), could modify expression of resistance and susceptibility alleles (**Figure 6-4**).

While the identities of the *Nicotiana*'s nepovirus resistance alleles are unknown, re-inoculation experiments (**Table 6-4**) show that the resistance signal is translocated to result in systemic recovery. Because the *N. tabacum* ToRSV resistance response appears to be SA-mediated (Jovel *et al.*, 2011), susceptibility alleles conferred by *N. benthamiana* in the 2x(*N. tabacum* x *N. benthamiana*) hybrid could allow ToRSV to quantitatively inhibit SA biosynthesis, affect conversion of SA to an alternate derivative, or vitiate downstream SA-activated resistance responses (Carr *et al.*, 2010; Boatwright and Pajeroska-Mukhtar, 2013). Similarly, null or ineffective RNA silencing alleles present in susceptible backgrounds could conceivably compromise RNA silencing-mediated virus resistance in hybrids (Incarbone and Dunoyer, 2013). These hypotheses are consistent with Fraser's model of virus

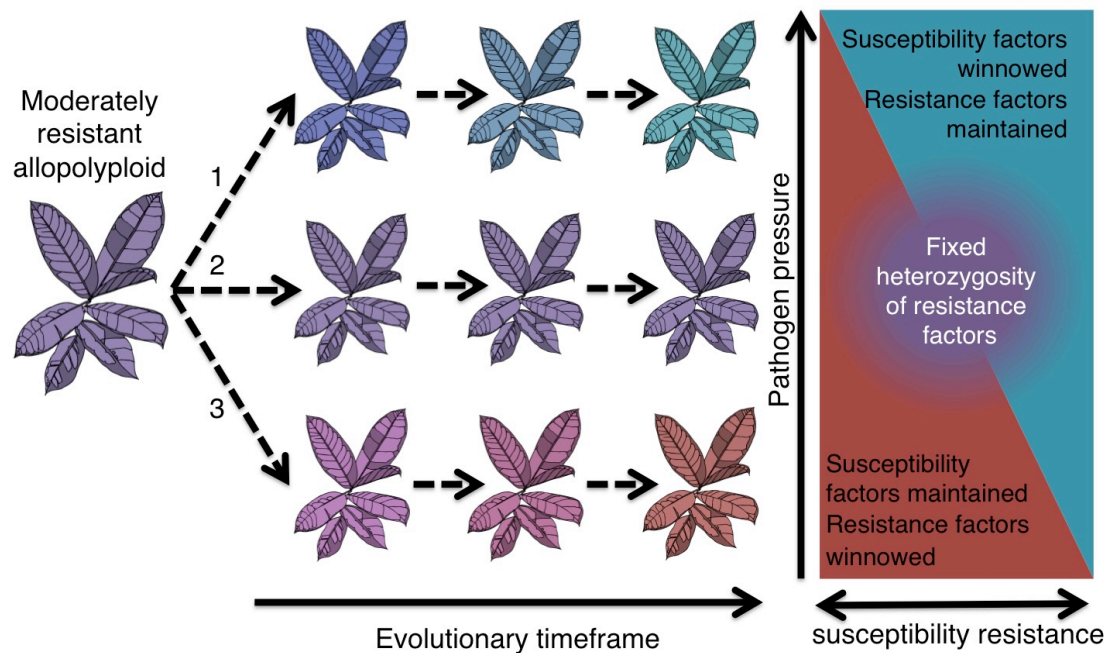


**Figure 6-4** Pictographic description of the modified matching allele model applied to the additive resistance hypothesis. Resistant and susceptible parents (e.g. diploid progenitors of an allopolyploid) carry unique complements of resistance factors (blue) and susceptibility factors (red). The allopolyploid plant would maintain a mix of resistance and susceptibility factors from each parent (fixed heterozygosity), and also would be expected to exhibit unique (nonadditive) expression profiles of resistance and susceptibility factors.

resistance (1992), which postulates that the effects of resistance alleles are proportional to their dosage and levels of influence on resistance pathways.

Although interspecific animal allopolyploids frequently show dominant parasite susceptibility (Moulia, 1999; Wolinska *et al.*, 2008; King *et al.*, 2012), *Nicotiana* neoallopolyploids exhibit virus resistance that is greater than one but not both of their parents (**Figure 6-3**). In cases where both parents were either resistant or susceptible, the neoallopolyploid displayed a resistance response similar to their parents, and thus there was no inherent penalty or benefit from hybridization or genome duplication (**Figure 6-3**). Contrary to the model that neoallopolyploid plants could face a depression of innate immunity (Jackson and Chen, 2010), our findings suggest that allopolyploidization itself did not penalize *Nicotiana* for virus resistance.

‘Revolutionary changes’ that accompany polyploidy can be distinguished from ‘evolutionary changes,’ which follow allopolyploidization (Feldman *et al.*, 2012; Soltis, 2013). The maintenance of virus resistance in *N. tabacum* contrasts with the apparent loss of virus resistance in section *Polydicliae*, which did not maintain partial virus resistance imparted by its likely maternal genome donor (*N. obtusifolia*) (**Figure 6-3**). Similarly, members of *Suaveolentes* exhibited high degrees of virus susceptibility despite the resistance of their paternal genome donor’s closest relative (*N. sylvestris*). Low virus resistance in sections *Polydicliae* and *Suaveolentes* suggests genetic drift and/or selection conferred a loss of virus resistance inherited by neoallopolyploids. *Nicotiana* neoallopolyploids show gene loss and neofunctionalization (Kovarík *et al.*, 2004; Clarkson *et al.*, 2005; Renny-Byfield *et al.*, 2011). Since favorable alleles have a lower chance of becoming fixed in



**Figure 6-5** Model of changes in quantitative innate virus resistance from a moderately resistant progenitor exhibiting fixed heterozygosity for resistance genes (e.g. a neoallopolyploid). Random divergence of the allopolyploid progeny leads to several possible lineages containing different resistance or susceptibility phenotypes whose existence depends on drift and pathogen pressure. High pathogen pressure would select for the loss of susceptibility factors and maintenance and gain of resistance factors (trajectory 1, top row). Moderate or irregular pathogen pressure would maintain an equilibrium of resistance and susceptibility factors within the plant population (trajectory 2, middle row). Low pathogen pressure would remove the selective advantage of maintaining resistance factors, and could result in the loss of resistance factors and the maintenance of susceptibility factors (trajectory 3, bottom row).

allopolyploids than diploids (Otto and Whitton, 2000), drift could result in losses of innate immunity alleles in the *Polydicliae* and *Suaveolentes* lineages (**Figure 6-5**).

The correlation of higher levels of virus resistance in South American *Nicotiana* species than North American and Australian species suggest that geographic influences had a major effect on the efficacy of antiviral resistance responses. Because natural *Nicotiana* neoallopolyploids exhibit an additive basal antiviral resistance (**Figure 6-3**), long-term biota-specific interactions would be critical factors to select for improved virus resistance. Existing virus resistance alleles could be maintained or enhanced if virus challengers perennially recur (trajectories 1 or 2), or virus resistance alleles could be lost if virus challenges diminish (trajectory 3) (**Figure 6-5**).

Comparison of several allopolyploids used in these virus resistance experiments suggest that *N. tabacum*, endemic to the relatively large and competitive biome of the Eastern Andes has followed trajectory 1, while members of sections *Suaveolentes* and *Polydicliae*, endemic to the relatively isolated biota of Australia and Southwest US, respectively, have followed trajectories 2 or 3 (**Figure 6-5**). Because *N. debneyi* and *N. benthamiana* are monophyletic (Lewis, 2011), but *N. debneyi* shows greater antiviral resistance than *N. benthamiana* (**Figure 6-3**), random or selection-based processes may have driven divergence of innate immune functions within this allopolyploid lineage. The sister allopolyploids *N. clevelandii* and *N. quadrivalvis* have similarly diverged for herbivory resistance responses (Lou and Baldwin, 2003).

Changes in immune function due to allopolyploidy could precipitate changes in challenging pathogens, and prompt a Red Queen-type evolutionary response between the plant and pathogen (Wolinska *et al.*, 2008). Ineffective innate immune



systems could allow otherwise ill adapted viruses to acquire more effective virulence factors and erode quantitative resistance (Vallad and Goodman, 2004; Palloix *et al.*, 2009). An allopolyploid that can endure colonization by a pathogen or pest and that permits pathogen adaptation to an otherwise resistant host progenitor is referred to as a hybrid bridge (Floate and Witham, 1993). Rather than escaping virus infection, allopolyploids could furnish another niche for viruses to expand their host ranges.

## **CONCLUSION**

The Red Queen Hypothesis explains how resistance and virulence temporally change in parasitic relationships. Allopolyploidy might represent an opportunity for plant hosts to break the Red Queen cycle of coevolution by gaining a new complement of dominant resistance factors, but the potential for allopolyploids to experience an epochal gain in innate immune function may be compromised by the inheritance of susceptibility alleles or genetic dysfunctionality caused by hybridization. The *Nicotiana*-nepovirus interaction sheds light on potential dynamics of how allopolyploidy may affect innate immunity. Based on a detailed survey of the interaction of non-coevolved plant and virus species, it appears that host and pathogen genotypes contain multiple alleles that interact in a quantitative fashion to determine the level of resistance or susceptibility. Synthetic allopolyploids faithfully display additive virus resistance characteristics that correspond to modified matching allele interactions (**Figure 6-4**). Virus resistance/susceptibility factors change in allopolyploid progeny due to classical drift and selection (**Figure 6-5**). These changes raise the interesting possibility that moderately resistant allopolyploids could provide a

hybrid bridge, which could result in a new Red Queen cycle of coevolution.

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## CHAPTER 7

### ***FUTURE DIRECTIONS***

#### ***NEPOVIRUS SUBGROUP A SATELLITE RNA***

Future studies on the GFLV satRNA could shed light on how viral RNAs are recognized and replicated specifically by RNA dependent RNA polymerases (RdRps). The GFLV satellite RNA encodes a highly basic protein (P3, comprised of isoelectric points ranging from 10.2 to 11.8), which does not have any known ontology or homology to other proteins. High pH proteins are known to be involved in interactions with nucleic acids, and the satellite RNA of *Bamboo mosaic virus* (satBaMV), a potexvirus, encodes a highly basic protein that specifically brings the *Bamboo mosaic virus* (BaMV) RdRp in contact with satBaMV (Tsai *et al.*, 1999; Palani *et al.*, 2009).

At the bare minimum, protein P3 of the GFLV satellite RNA is expected to effect its replication. Quite possibly the GFLV satellite RNA may confer specific selective advantages that could result in its acquisition and maintenance by GFLV and ArMV strains. The interactors of the GFLV satellite RNA could be identified with immunoprecipitation assays, yeast two-hybrid assays or yeast three-hybrid assays (with the satellite RNA used as a link between bait and prey). Additionally, Moser *et al.* (1992) tentatively identified protein P3 in the nuclei of infected cells, which could suggest the satellite RNA interacts with DNA as a transcriptional regulator. To test the transcriptional regulator theory, a chromatin immunoprecipitation (ChIP) assay could be designed to target the P3 protein using nanobodies followed by DNA purification

and sequencing or microarray analysis (Nguyen-Duc *et al.*, 2012). Identifying the host- and virus-targets of P3 should shed light on how the satellite RNA is able to be specifically replicated by its helper virus, and could reveal its functional significance.

Viral satellites, including satellite RNAs, can alter virus host range (Zhou *et al.*, 2013), modulate symptoms (Shimura *et al.*, 2011), or regulate viral RNA silencing suppression (Thomas *et al.*, 2003). According to this author's research (Chapter 2) and the research of others (Saldarelli *et al.*, 1993; Lamprecht *et al.*, 2013), the satellite RNA of GFLV does not affect infected grapevines or *C. quinoa*. Because the Lilac satellite of *Arabis mosaic virus* (ArMV) ameliorates symptoms in *C. quinoa* (Liu *et al.*, 1991), it could be interesting to test whether this is due to an interaction with the helper virus (i.e. GFLV versus ArMV) or a satellite genotype-specific response. The ultimate test of satellite RNA pathological impact would be an experiment in grapevine, the primary natural host of GFLV. Experiments are underway at INRA Colmar, France to test the pathological impact of various GFLV strains, including some with a satellite RNA, in several cultivars (E. Vigne, personal communication). This information could yield clues about virulence mechanisms of GFLV and could facilitate the design of disease control strategies (Collmer and Howell, 1992).

The incomplete discovery of the origin of the nepovirus subgroup A satellite RNA (Chapter 2) opens new questions in virus evolution. The nepovirus subgroup A satellite RNA appears to have originated as a recombination event between an ancestral nepovirus genomic RNA and another unknown RNA. This most likely would have occurred during positive-strand synthesis of the genomic RNA, where a template switch by the viral RdRp resulted in recombination between the 5' end of the

viral genomic RNA and the 3' end of a pathogen or host RNA. Although the mechanics of nepovirus replication are currently poorly understood, it is possible that nepoviruses behave like other viruses with genome-linked proteins (VPgs) and poly-A tails (e.g. potyviruses) in that they use the translation initiation complex to anchor for replication (Lellis *et al.*, 2002). Proximity of heterologous templates bound to the translation initiation complex could provide the opportunity for recombination. The identities of host proteins and membranes interacting to form nepovirus replication complexes could shed light on mechanistic features of nepovirus recombination (Jiang and Laliberté, 2011; Sanfaçon *et al.*, 2012), including that of the subgroup A satellite RNA.

The recombination event that gave rise to the satellite RNA resulted in a truncation of its 5' untranslated region (UTR) and an early start codon. The start codon lies after a 14 to 18 nucleotide UTR, which is sufficient for eukaryotic translation initiation (Watson *et al.*, 2007). The satellite RNA has seven conserved blocks of nucleotides that extend 140 nucleotides from its 5' end. This feature suggests it has maintained the RNA structural elements necessary for VPg, RdRp and capsid recognition. This overlap of protein coding- and RNA structural regions suggests dual selective mechanisms are at work on the 5' 140 nt of the satellite RNA, and indeed the N-terminus of P3 possesses biochemical properties atypical of the rest of the protein, including high hydrophobicity and an overabundance of positively charged amino acids (Fuchs *et al.*, 1989). The nepovirus subgroup A satellite RNA's conservation of seven groups of 5' nucleic acid stretches point to the elements that are needed for viral RNA recognition by viral proteins. Comparative and mutagenic studies on the 5'

nucleotides of the satBaMV have yielded an abundance of information about its *cis*- and *trans*-replication elements (Annamalai *et al.*, 2003; Chen *et al.*, 2012) and similar research projects could be envisioned for the GFLV satellite RNA.

The identity and source of the gene that comprises the 3' end of the GFLV satellite RNA is unknown. There is currently no parameter on NCBI BLAST that shows molecules with significant protein or nucleic acid homology to the protein of the satellite RNA. This could be because the satellite RNA has diverged significantly from its progenitor, or because the gene has not been sequenced or deposited in NCBI. More publically available sequences from multiple organisms will increase the likelihood that genes with significant homology will be found. Because viruses are estimated to contain 50% of protein diversity on earth (Holmes, 2009), there is a high probability that the recombinant satellite RNA gene came from a virus.

Sequencing more GFLV isolates including targeted amplification of the satellite RNA ought to shed light on the natural history of GFLV with respect to anthropogenic interactions. Humans have distributed GFLV worldwide, and high levels of diversity are often found inside single vineyards (Mekuria *et al.*, 2009; Oliver *et al.*, 2010). High intra-population diversity coupled with numerous instances of recombination makes it difficult to trace the origin of particular strains to a site of origin. The satellite RNA, on the other hand, is less efficiently transmitted from plant-to-plant by *X. index* than its helper virus (Chapter 2). This means that the satellite RNA could function as a 'stable biomarker' that can be used to trace the origin of particular strains. For example, the presence of a common satellite RNA in a grapevine in Bordeaux and a vineyard in California suggests a very recent common



origin of that satellite RNA variant (Chapter 2).

Using the satellite RNA as a viral vector for plant functional genomics could be an interesting proposition because it could provide higher levels of expression than the genomic RNAs. Although this author attempted to use the GFLV satellite RNA as a vector, it was non-infectious. Lamprecht *et al.* (2013) designed a construct based on the GFLV satellite RNA SACH44 using a pBluescript vector where a CaMV 35S promoter drove expression of the satellite RNA and the vector was rub-inoculated to a *C. quinoa* mechanically inoculated with sap containing GFLV, and the satellite RNA was replicated. It could be interesting to see if this infectious satellite RNA could stably replicate and express heterologous genes such as fluorophors, or if any modification will abolish its ability to be replicated. Agroinfection is the unattained ideal for a GFLV vector for grapevine, so the next logical step would be to attempt agroinfection of the GFLV genomic RNAs with the satellite RNA.

### ***IMPROVING VIRAL VECTORS***

Plant virus vectors share few commonalities, so there are few generalizable improvements that can be prescribed to all plant virus vectors. The main aspect that needs attention is the fidelity of the heterologous virus sequence. Infectious transcripts are key to developing RNA viral vectors, but frequently cDNA copies of viral RNA transcripts are not infectious (Liu *et al.*, 2009; Youssef *et al.*, 2011, Kurth *et al.*, 2013). The cDNAs could be noninfectious because of the error-prone nature of reverse transcriptases (RTs) or that non-infective variants of RNAs are amplified in subsequent PCR (Chapman, 2008). Strategies to generate infectious clones of RNA

viruses are generally more successful when multiple clones are tested and high fidelity RTs and DNA polymerases are used (Youssef *et al.*, 2011).

DNA viruses and RNA viruses have been engineered into viral vectors and the route of delivery varies based on the nucleic acid identity of the virus. DNA viruses may be amplified by PCR and circularized by DNA ligation and directly inoculated to plants or agroinoculated if the virus is imparted with an *A. tumefaciens* origin of replication (Huang *et al.*, 2009). Positive-sense RNA viral vectors can be delivered to plants through inoculation of *in vitro* transcripts or transcription driven by the CaMV 35S promoter which include biolistic inoculation, direct plasmid rub-inoculation or agroinfection (Robertson, 2004). Negative sense RNA viruses can be agroinoculated to plants as long as the necessary replicase proteins are co-inoculated (Ganesan *et al.*, 2013). The optimal method of virus vector inoculation depends on the genomic structure of individual viruses and is best determined empirically.

RNA-based viral vectors can be designed for *in planta* transcription by the CaMV 35S promoter or other promoters. Lim *et al.* (2010) developed a novel *in planta* promoter based on the T7 phage RNA polymerase (T7RNAP) to express *Alternanthera mosaic virus*. This strategy placed the viral cDNA upstream of the T7 promoter and co-expressed the T7RNAP protein via the CaMV 35S promoter. These researchers showed that the T7 system shortens the time to systemic infection from two weeks to seven days (Lim *et al.*, 2010). Similar benefits were achieved when *Tobacco mosaic virus* (TMV), another RNA virus, was expressed by an RNA polymerase I promoter (Komarova *et al.*, 2012a). It is notable that because the capping, splicing and polyadenylation machinery is transcriptionally interconnected

with RNA polymerase II function (Watson *et al.*, 2007), transcripts corresponding to viral RNAs would not be modified in the same way as messenger RNAs if expressed via T7RNAP or RNA polymerase I.

Another way to avoid splicing is to mutate ‘cryptic introns’ encoded by the viral cDNA, i.e. to change putative acceptor and donor sites that could form the extremities of an intron (Marillonet *et al.*, 2005; Komarova *et al.*, 2012b). Ideally, the mutation of cryptic introns can be accomplished through silent mutations so that functionality of the viral proteins will not be affected. Alternatively, placing heterologous plant introns within the viral sequence can diminish splicing of cryptic introns within the viral cDNA (Marillonet *et al.*, 2005). Because changing the predicted splicing characteristics of viral vector RNAs has only been accomplished with TMV, it remains to be seen how generalizable the observed expression enhancements to RNA viruses can be.

Because RNA viruses vary in types of 5’ and 3’ genomic modifications (e.g. 5’ caps, genome-linked proteins, poly-A tails or cloverleaf structures), optimal viral RNA expression strategies should depend on the nature of the viral RNA. In general, researchers should avoid using certain promoters just because they are readily available, but instead give sober consideration to expression of a transcript as close as possible to authentic viral RNA.

Viral RNAs corresponding to about 60 virus species have been transiently expressed *in planta* using diverse methods of inoculation (Senthil-Kumar and Mysore, 2012). Most have relied on the CaMV 35S promoter following rub inoculation or agroinoculation of the plasmid and/or its T-DNA, respectively. Particle bombardment

or transfection of protoplasts are also commonly used to express a CaMV 35S-driven RNA transcript. Several binary vectors have been used to express viral RNAs following agroinoculation including pBIN (Ratcliff *et al.*, 2001; Agüero *et al.*, 2012; Delfosse *et al.*, 2013), pGreen (Ratcliff *et al.*, 2001; Larsen and Curtis, 2012; Bedoya *et al.*, 2012), and pCAMBIA (Muruganantham *et al.*, 2009; Lim *et al.*, 2010). Though pGA482 has been used to express individual proteins of *Turnip vein clearing virus* (Harries *et al.*, 2009) and *Potato virus X* (Larsen and Curtis, 2012) upstream of a CaMV 35S promoter with a heterologous translational enhancer sequence, this author is not aware of any published viral vectors that used pGA482-derived binary vectors. The GFLV vector (Chapters 3 and 4) uses pGA482 for agroinoculation with the CaMV 35S expression cassette from pEPT8. The plasmid EPT8 contains a translational enhancer sequence derived from the 5' end of *Alfalfa mosaic virus* (AIMV) RNA4, which forms a hairpin (Ling *et al.*, 1997). Because the 5' ends of viral genomic RNAs are essential for RNA-RdRp interactions (Annamalai *et al.*, 2003; Chen *et al.*, 2012), the heterologous hairpin tagged to the 5' end of the GFLV genomic RNA could possibly interfere with its RNA-RdRp interactions. Notably, GFLV vectors that use the same viral cDNAs as those described in Chapters 3 and 4 (F13 RNA1 plus *Green fluorescent protein* or *Red fluorescent protein*-tagged F13 RNA2) but expressed in vectors that do not tag the 5' ends of transcripts with the AIMV hairpin (Chapter 4) show 100% systemic infection in *N. benthamiana*, whereas the GFLV-eGFP and the GFLV-RFP chimeras containing AIMV 5' hairpin result in 0% and 1.6% plant infection in populations of 60 to 100 plants, respectively (Gottula and Keichinger, unpublished results). These results suggest that using a protein expression

vector (i.e. a vector that imparts a heterologous virus hairpin molecule for translation enhancement) is not the ideal vector to deliver infectious RNA transcripts *in planta*. It will be interesting to see if functional GFLV cDNAs without heterologous 5' hairpins and/or fused protein reporters will be systemically infectious in grapevine.

### ***THE FUTURE OF PLANT VIRUS RESISTANCE***

Plant viruses were first described by Beijerinck (1892) who discovered a subcellular agent (TMV) is responsible for a mosaic on tobacco. Since that time, TMV has been a model for plant-virus interactions. Resistance to TMV was discovered in *N. glutinosa* and the single-gene source of resistance was introgressed into *N. tabacum* (Lewis, 2011). The *N* gene has since been characterized on a cell and molecular basis and typifies single gene sources of resistance in that it encodes for an NB-LRR protein which induces hypersensitive responses in a temperature-dependent manner (Kang *et al.*, 2005). Single gene sources of resistance can be durable, especially against viral pathogens (Fraile and García-Arenal, 2010), but are generally less durable than quantitative, multigenic types of resistance (McDonald, 2009). Plant virus resistance genetics ranging from single gene dominant, to quantitative and single gene recessive have been reported (Palukaitis and Carr, 2008). Single gene sources of resistance are typically qualitative and race/strain-specific, while quantitative types of resistance form non-race-specific defenses against whole classes of invading pathogens. The latter may be termed basal resistance (Jones and Dangl, 2006)

Quantitative resistance to viruses is difficult to introgress into breeding populations and can be complicated to study. It usually does not confer immunity but

instead partial resistance that is not race dependent (Palloix *et al.*, 2009). One manifestation of quantitative virus resistance is recovery from infection, where plant viruses can infect part of the plant but resistance is acquired in other parts of the plant system (Cooper and Jones, 1983; Bruening, 2006). Although recovery from virus infection is frequently associated with hypersensitive reaction (HR), reverse and forward genetic evidence has decoupled the HR response and recovery from virus infection (Kim and Palukaitis, 1997; Bendahmane *et al.*, 1999; Cole *et al.*, 2001). Cole *et al.* (2001) demonstrated that HR and recovery from CaMV strain W260 infection were separate phenomena governed by separate genes in segregating interspecific hybrids of *N. clevelandii* and *N. glutinosa*.

Host genotypic effects, viral and environmental factors govern recovery from infections. Recovery from plant virus infections may be due to dominant genes such as *DSTM1* in *Arabidopsis* and *Wmv* in bean (Serrano *et al.*, 2008; Kyle and Provvidenti, 1987), incompletely dominant genes such as three *RTM* genes in *Arabidopsis* and *Tm-1* in tomato (Chisholm *et al.*, 2001; Cosson *et al.*, 2010; Ishibashi *et al.*, 2007), or recessive genes such as tomato *tm-1* transgenically expressed in tobacco, *ra* in potato and *vsm-1* in *Arabidopsis* (Ishibashi *et al.*, 2009; Hämäläinen *et al.*, 2000; Lartey *et al.*, 1998). Quantitative virus resistance genes can also confer recovery from virus infection (Chandra-Shekara *et al.*, 2004; Kang *et al.*, 2005; Maule *et al.*, 2007). Viral virulence factors can compromise *Rsv4*-dependent recovery from *Soybean mosaic virus* in soybean (Gunduz *et al.*, 2004), and CaMV recovery in *N. edwardsonii* (Cole *et al.*, 2001). Temperature and gene dosage positively regulates *I* gene-mediated *Bean common mosaic virus* recovery in bean (Collmer *et al.*, 2000), and temperature

negatively regulates recovery from *Tobacco ringspot virus* (TRSV) in *N. tabacum* (Siddiqui *et al.*, 2008). Recovery from virus infection appears to be due to a quantitative interaction influenced by host genetics, virus virulence and environment.

Quantitative resistance is generally more durable, less pathogen race/strain-specific, and more stable at a wider range of environmental conditions than NB-LRR types of resistance (Boiteux *et al.*, 2012; Robinson, 2007). In the age of whole genome sequencing, minichromosomes, and high-throughput phenotypic resources, studying and selecting for quantitative resistance has become more feasible than ever.

Pathogen-derived resistance (PDR) shares features with both single-gene qualitative resistance and quantitative/basal resistance. PDR is like single gene resistance in that it is usually conferred by a single transgene, and thus may be easily selected during breeding. PDR can also confer qualitative resistance that has been observed, for example in breakdowns of resistance in the SunUp papaya to *Papaya ringspot virus* isolates that are highly diverged from the Hawaiian strain from which the transgene was sourced (Chapter 5). PDR can behave more like qualitative resistance in that it can confer stable resistance to multiple virus strains if the transgene is designed in conserved regions of virus genomes (Chapter 5). Thus PDR can capture many of the advantages of single gene resistance (for breeding using single gene introgression) while also maintaining the durability of resistance that accompanies quantitative resistance.

The future of plant virus resistance will be determined through advanced understanding of plant-virus interactions. Breeders should select varieties that are tested over long periods of time in multiple locations with high and uniform levels of

pathogen pressure (Robinson, 2007). Interspecific hybridization will play a role in introgressing quantitative sources of virus resistance into crops provided barriers to recombination and reduced hybrid vigor can be overcome (Thurston, 1961). PDR will be of greatest use when designed to target conserved regions of viral genomes and tested over long periods of time in multiple locations. The ideal strategy to create durable virus resistance will involve stacking quantitative and pathogen-derived resistance genes to form a multi-layered barrier to virus infection. Quantitative resistance and PDR together is the best way to achieve long-lasting and stable virus resistance in crops.



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